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The Effects of TCA Cycle Mutations on the Virulence of Vibrio Aguillarum Strains M93SM and NB10SM

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THE EFFECTS OF TCA CYCLE MUTATIONS ON THE VIRULENCE OF VIBRIO ANGUILLARUM STRAINS M93SM AND NB10SM.

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ABSTRACT

Vibrio anguillarum is an extracellular bacterial pathogen that is a causative agent of vibriosis in finfish and crustaceans. Mortality rates range from 30% to 100% and systemic infection usually causes fish to die within 1-4 days of initial infection. The primary routes of infections are through the skin, gills and intestines. Chemotactic motility and the metalloprotease EmpA have been shown to be important virulence factors during the invasion stage while the siderophore anguibactin, flagellin subunits and lipopolysaccharides were shown to be important for persistence in the host during the post-invasion stage. Three secreted proteins that are cytotoxic against epithelial cells and erythrocytes have been characterized in *V. anguillarum*: the HlyA homolog Vah1, the phospholipase Plp, and the MARTX toxin RtxA. Previous research has demonstrated that mutations in *vah1* and/or *plp* resulted in slight attenuation against juvenile Atlantic salmon (*Salmo salar*); however, *rtxA* mutants were avirulent. Expression of the cytotoxins are under control of the transcriptional activator HlyU and the repressor H-NS. Additionally, a *V. anguillarum hns* mutant showed attenuation in virulence when injected intraperitoneally, suggesting that proper coordination of gene expression is an important factor during the post-invasion stage.

In manuscript I "Isocitrate dehydrogenase mutation in *Vibrio anguillarum* results in virulence attenuation and immunoprotection in rainbow trout (*Oncorhynchus mykiss)*", seven central metabolism mutants were created in the M93Sm strain and characterized with regard to growth in minimal and complex media, expression of virulence genes and virulence in juvenile rainbow trout. Only the isocitrate dehydrogenase (*icd*) mutant was attenuated in virulence against rainbow trout challenged by either intraperitoneal

injection or immersion. Further, the *icd* mutant was shown to be immunoprotective against wild type *V. anguillarum* experimental challenge. The *icd* mutant did not demonstrate a significant decrease in the expression of the three hemolysin genes was detected by qRT-PCR. Only the *icd* mutant exhibited a significantly decreased growth yield in complex media that was directly related to the amount of glutamate. A strain with a restored wild type *icd* gene was created and shown to restore growth to a wild type cell density in complex and minimal media and pathogenicity in rainbow trout. The data strongly suggest that a decreased growth yield, resulting from the inability to synthesize α-ketoglutarate derivatives (glutamate and glutamine), caused the attenuation despite normal levels of expression of virulence genes. Therefore, the ability of an extracellular pathogen to cause disease may be dependent upon the availability of host-supplied nutrients for growth.

In manuscript II "Characterization of the growth and virulence of a *Vibrio anguillarum* citrate synthase mutant", the role of glutamate auxotrophy during *V. anguillarum* M93Sm infection was further characterized. A citrate synthase (*gltA)* deletion mutant was created and characterized with regard to growth in minimal and complex media, expression of virulence genes, and virulence in juvenile rainbow trout. The Δ*gltA* mutant exhibited a decreased final cell density when grown in LB20 that resulted from the exhaustion of glutamate from the media. There was no significant decrease in the expression of the three hemolysin genes by the Δ*gltA* mutant when detected by qRT-PCR or mortality during challenge experiments. A Δ*gltA* mutant capable of growing in minimal media was isolated and shown to have a spontaneous mutation in the transcriptional activator of 2-methylcitrate synthase (*prpR*). This mutation

resulted in an increase in expression of 2-methylcitrate synthase (*prpC*). The Δ*gltA prpR*(R66L) mutant was characterized with regard to growth in complex media and exhibited a growth advantage compared to the Δ*gltA* mutant after 24 h in spleen extract medium. Further, after growing 120 h in spleen extract medium, colonies of Δ*gltA* mutants were shown to be capable of growing in minimal media. Δ*prpC* and a Δ*gltA* Δ*prpC* mutants were created and characterized with regard to growth in minimal and complex media and virulence in juvenile rainbow trout. The Δ*gltA* Δ*prpC* mutant had no growth advantage in spleen extract medium compared to the Δ*gltA* but was still as virulent as the wild type against rainbow trout. As expected, the Δ*prpC* mutant was similar to the wild type in regards to both growth in minimal and complex media and virulence against rainbow trout. The data strongly suggests that simple starvation for α-ketoglutarate derivatives (glutamate and glutamine) is not directly linked to attenuation of virulence as previously proposed. Additionally, spontaneous mutations can occur that compensate for the original gene deletion if the new mutation can replace or bypass the lost metabolic reaction and results in a growth advantage.

In manuscript III "Characterization of *Vibrio anguillarum* NB10Sm TCA cycle mutants" the role of central metabolism in virulence was examine in the O1 serotype strain of *V. anguillarum* NB10Sm. A *V. anguillarum* NB10Sm *icd* mutant was created, characterized for growth in complex media and demonstrated to be as virulent as the wild type in juvenile rainbow trout. Several additional central metabolism single and double mutants were created in the following genes *cra, gltA, Δicd gltA, sucA, sucC, sdhC, ΔfrdA, ΔfrdA sdhC,* and *fumA* and characterized with regard to growth in complex media. Two mutants (Δ*sucA* and Δ*frdA* Δ*sdhC*) that demonstrated a significantly reduced growth

yield compared to the wild type were further characterized with regard to their growth in several forms of complex media, expression of virulence genes, and virulence in juvenile rainbow trout. The data strongly suggest that there is no correlation between a lower growth yield *in vitro* and a decrease in virulence *in vivo*. Even though M93Sm and NB10Sm are same species, mutations made in the same TCA cycle genes can cause drastically difference results in regards to growth and virulence.

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PREFACE

This dissertation has been prepared in the Manuscript format according to the guidelines of the Graduate School of the University of Rhode Island. The first manuscript "Isocitrate dehydrogenase mutation in *Vibrio anguillarum* results in virulence attenuation and immunoprotection in rainbow trout (*Oncorhynchus mykiss*)" was submitted to *BMC Microbiology* July 31st 2017 and is under revision. The second manuscript "Characterization of the growth and virulence of a *Vibrio anguillarum* citrate synthase mutant" and the third manuscript "Characterization of *Vibrio anguillarum* NB10Sm TCA cycle mutants" will be submitted for publication to *BMC Microbiology*. The first appendix "Draft genome of the marine pathogen *Vibrio coralliilyticus* RE22" was published Dec 3rd 2015 in Genome Announcements. The second appendix "Draft genome sequence of the emerging bivalve pathogen *Vibrio tubiashii* subsp. europaeus" was published July 28th 2016 in Genome Announcements.

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Isocitrate dehydrogenase mutation in *Vibrio anguillarum* results in virulence attenuation and immunoprotection in rainbow trout (*Oncorhynchus mykiss)*

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Abstract

Background: *Vibrio anguillarum* is an extracellular bacterial pathogen that is a causative agent of vibriosis in finfish and crustaceans with mortality rates ranging from 30% to 100%. Mutations in central metabolism (glycolysis and the TCA cycle) of intracellular pathogens often result in attenuated virulence due to depletion of required metabolic intermediates; however, it was not known whether mutations in central metabolism would affect virulence in an extracellular pathogen such as *V. anguillarum*.

Results: Seven central metabolism mutants were created and characterized with regard to growth in minimal and complex media, expression of virulence genes, and virulence in juvenile rainbow trout (*Oncorhynchus mykiss*). Only the isocitrate dehydrogenase (*icd*) mutant was attenuated in virulence against rainbow trout challenged by either intraperitoneal injection or immersion. Further, the *icd* mutant was shown to be immunoprotective against wild type *V. anguillarum* infection. There was no significant decrease in the expression of the three hemolysin genes was detected by qRT-PCR. Additionally, only the *icd* mutant exhibited a significantly decreased growth yield in complex media. Growth yield was directly related to the abundance of glutamate. A strain with a restored wild type *icd* gene was created and shown to restore growth to a wild type cell density in complex media and pathogenicity in rainbow trout.

Conclusions: The data strongly suggest that a decreased growth yield, resulting from the inability to synthesize α-ketoglutarate, caused the attenuation despite normal levels of expression of virulence genes. Therefore, the ability of an extracellular pathogen to cause disease is dependent upon the availability of host-supplied nutrients for growth.

Additionally, a live vaccine strain could be created from an *icd* deletion strain.

Keywords: *Vibrio anguillarum*, TCA cycle, vibriosis, isocitrate dehydrogenase, virulence, hemolysin

Background

The aquaculture industry now produces half of all fish intended for human consumption and employs millions of people worldwide [1]. Although the first value sale of harvested fish has increased by 267% between 2004 and 2014 to over US$160 billion, infectious diseases, especially those caused by *Vibrio* spp. including *Vibrio anguillarum*, still represent a major impediment to the production of fish [1]. *V. anguillarum* causes diseases in crustaceans and bivalves, and is the leading causative agent of vibriosis in finfish including salmon, rainbow trout, turbot, sea bass, sea bream, cod, eel, and ayu [2]. Infections by this bacterial species have resulted in severe economic losses to aquaculture industries worldwide [3].

V. anguillarum is an extracellular pathogen that invades its host fish through the intestine, skin or gills [4, 5]. Systemic infection by *V. anguillarum* usually causes fish to die within 1-4 days [6-9]. Chemotactic motility and the metalloprotease EmpA have been shown to be important virulence factors during the invasion stage while the siderophore anguibactin, flagellin subunits and lipopolysaccharides were shown to be important for persistence in the host during the post-invasion stage [2, 10]. Three secreted proteins that are cytotoxic against epithelial cells and erythrocytes have been characterized in *V. anguillarum*: the HlyA homolog Vah1, the phospholipase Plp, and the MARTX toxin RtxA [7, 9, 11]. Mutations in *vah1* and/or *plp* resulted in slight attenuation against juvenile Atlantic salmon (*Salmo salar*); however, *rtxA* mutants were avirulent [11, 7, 9]. Additionally, a *V. anguillarum* mutant that lacks H-NS, a global transcriptional regulator that represses the transcription of *vah1*, *plp*, and *rtxA*, showed attenuation in virulence

when injected intraperitoneally, suggesting that proper coordination of gene expression is an important factor during the post-invasion stage [8].

Since the 1980s, several bacterial species that are auxotrophic for aromatic compounds have been shown to be avirulent [12-16]. More recently, mutants that are hypothesized to experience growth defects in the nutrient limited environment inside a phagocyte have been characterized. In *Salmonella enterica*, an intracellular bacterial pathogen, some tricarboxylic acid (TCA) cycle mutant strains were avirulent and immunoprotective for subsequent wild-type *S. enterica* infection [17-21]. A functional fructose repressor (Cra) was also required for *S. enterica* infection [22]. Similar results have been observed for central metabolism mutants in other intracellular pathogens such as uropathogenic *Escherichia coli* (UPEC), *Mycobacterium tuberculosis*, and the facultative intracellular fish pathogen *Edwardsiella ictaluri* [23-27]. These observations demonstrate that central metabolism is important for pathogenesis by intracellular pathogens.

Accordingly, we hypothesized that mutations in central metabolism could interrupt the infection process of *V. anguillarum* in juvenile rainbow trout *(Oncorhynchus mykiss)*. In this study, we identified and created six TCA cycle mutant strains plus one fructose metabolism mutant strain, and tested their virulence against juvenile rainbow trout using two infection methods, intraperitoneal (IP) injection and immersion. Further, the expression of each of the three hemolysin genes (*vah1*, *plp*, and *rtxA*) was examined to determine whether attenuation resulted from decreased virulence factor expression in these mutants. The growth rates and yield of each mutant strain in complex media were also determined. We specifically characterized the growth defect of the attenuated *icd*

mutant. We also created, tested, and compared a restored wild type *icd* strain for virulence and growth to both the wild type and the *icd* mutant.

Methods

Bacterial strains, plasmids and growth conditions. *V. anguillarum* strains (Table 1) were routinely grown in Lysogeny broth containing 2% NaCl (LB20) [28] or Marine Minimum Median (3M) + 0.15% glucose [29], supplemented with the appropriate antibiotic, in a shaking water bath at 27°C. *E. coli* strains (Table 1) were routinely grown in Lysogeny broth containing 1% NaCl (LB10) supplemented with the appropriate antibiotic, in a shaking water bath at 37°C. Antibiotics were used at the following concentrations: streptomycin, 200 μg/ml (Sm^{200}); chloramphenicol, 20 μg/ml (Cm^{20}) for *E. coli* and 5 μg/ml (Cm^{5}) for *V. anguillarum*; kanamycin, 50 μg/ml (Km^{50}) for *E. coli* and 80 μg/ml (Km^{80}) for *V. anguillarum.*

Identification of genes in *V. anguillarum*. *V. anguillarum* M93Sm draft genome (accession number NOWD00000000) was annotated by the RAST (Rapid Annotation using Subsystem Technology) service (http://rast.nmpdr.org/rast.cgi) using the default settings [30]. The following annotated genomes were downloaded from NCBI: *V. anguillarum* 775 (accession numbers: NC_015633.1 and NC_015637.1), 96F (accession number: NZ_AEZA00000000.1), M3 (accession numbers: NC_022223.1, NC_022224.1 and NC_022225.1), NB10 (accession numbers: NZ_LK021130.1, NZ_LK021129.1 and NZ_LK021128.1), RV22 (accession number: AEZB00000000.1) and 90-11-286 (accession numbers: NZ_CP011460.1 and NZ_CP011461.1)

Insertional mutagenesis. Insertional mutations were made by using a modification of the procedure described by Milton *et al.* [31]. Briefly, primers (Table 2) were designed based

on the target gene sequence of M93Sm. An internal 200-300 bp DNA fragment of the first third of the target gene was PCR amplified and ligated into the suicide vector pNQ705-1 (Table 1) after digestion with SacI and XbaI. The ligation mixture was introduced into *E. coli* SM10 by electroporation using a BioRad Gene Pulser II (BioRad, Hercules, CA). Transformants were selected on LB10 Cm^{20} agar plates. The construction of the recombinant pNQ705 was confirmed by both PCR amplification and restriction enzyme analysis. The mobilizable suicide vector was transferred from *E. coli* SM10 into *V. anguillarum* by conjugation [32]. Transconjugants were selected by utilizing the chloramphenicol resistance gene located on the suicide plasmid. The incorporation of the recombinant pNQ705 was confirmed by PCR amplification.

Fish infection experiments. Various *V. anguillarum* strains were tested for virulence against rainbow trout *(O. mykiss)* by intraperitoneal (IP) injection or immersion. Briefly, *V. anguillarum* cells grown for 19 h at 27°C in LB20 supplemented with the appropriate antibiotics were harvested by centrifugation (9,000 × *g*, 5 min, 4°C), washed twice in NSS, and resuspended in NSS. Aliquots (100 µl) of the *V. anguillarum* NSS suspension were used to determine the OD_{600}. The *V. anguillarum* NSS suspension was prepared to the desired specific cell density according to the conversion equation as determined by experimentation (data not shown): Cell density (10^8 CFU/ml) = 44.905 × OD_{600}. The actual cell density of the suspension was confirmed by dilution and viable plate count. All fish were examined and determined to be disease and injury free prior to the start of each experiment. For IP injection, fish were anesthetized by tricaine methanesulfonate (Western Chemical, Ferndale, WA), (100 mg/l for induction and 52.5 mg/l for maintenance). *V. anguillarum* strains were IP injected into fish that were

between 15 and 25 cm long in a 100 µl NSS vehicle at a dose of either 2 × 105 or 4 × 105 CFU/fish, or with NSS only as a negative control. For immersion, 10 ml of *V. anguillarum* suspended in NSS, or 10 ml of NSS only as a negative control was added to a bucket filled with 10 L of water supplemented with 1.5% NaCl that was maintained at 18 ± 1ºC. Fish that were between 15 and 25 cm long were added and immersed for 1 h. For both methods, fish inoculated with different bacterial strains were maintained in separate 10-gallon (38 L) tanks to prevent possible cross-contamination with constant water flow (200 ml/min) at 18 ± 1ºC. Death due to vibriosis was determined by the observation of gross clinical symptoms and confirmed by the recovery and isolation of *V. anguillarum* cells resistant to the appropriate antibiotics from the spleen or head kidney of dead fish. Observations were made for 8-14 days. All fish used in this research project were obtained from the URI East Farm Aquaculture Center. All fish infection protocols were approved by the URI IACUC. (IACUC Protocol AN06-08-002).

RNA isolation. Exponential phase cells (~0.5×10^8 CFU/ml) of various *V. anguillarum* strains were treated with RNAprotect Bacteria Reagent (QIAGEN), following the manufacturer's instructions. Total RNA was isolated using the RNeasy kit and QIAcube (QIAGEN) following the instructions of the manufacturer. All purified RNA samples were quantified spectrophotometrically by measuring absorption at 260 nm and 280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and overall quality was assessed by gel electrophoresis. Samples were stored at -75ºC for future use.

Real-time quantitative RT-PCR (qRT-PCR). qRT-PCR was used to quantify various mRNAs using an LightCycler® 480 Real-Time PCR System (Hoffmann-La Roche Inc.) and the Brilliant II SYBR Green Single-Step QRT-PCR Master Mix (Agilent

Technologies), with 10 ng of total RNA in 20 µl reaction mixtures. The thermal profile was 50°C for 30 min, 95°C for 15 min, and then 40 cycles of 95°C for 30 s and 55°C for 30 s. Fluorescence was measured at the end of the 55°C stage of each cycle. Samples were run in triplicate along with the no-reverse-transcriptase control and the no-template control. All experiments were repeated at least twice.

Growth experiments. To cultivate bacteria for growth experiments, *V. anguillarum* cells grown overnight at 27°C in LB20 supplemented with the appropriate antibiotics were harvested by centrifugation (9,000 × *g*, 2 min), washed twice and resuspended in in NSS. A 200 µl aliquot of the *V. anguillarum* NSS suspension was transferred into a 96-well plate with a clear flat bottom and the optical density at 600 nm (OD_{600}) was read by a VersaMax™ Absorbance Microplate Reader (Molecular Devices). The *V. anguillarum* NSS suspension was prepared to an OD_{600} of 0.420 (~4×10^7 CFU/ml) and diluted 1:100 into fresh media. Growth was monitored either by measurement of the OD_{600} or by serial dilution and plate counts.

Resolving the merodiploid in the *icd* mutant. *V. anguillarum icd* mutant cells grown in LB20 supplemented with appropriate antibiotics for 19 h at 27°C were harvested by centrifugation (9,000 × *g*, 2 min), washed three times in NSS, and resuspended in NSS. Cell suspensions (100 µl) were spread onto Marine Minimum Median (3M) + 0.15% glucose agar. Well-isolated colonies were picked and subsequently streak purified onto a new 3M + 0.15% glucose agar. Isolated colonies were then transferred to LB20-Cm^5 agar to screen for chloramphenicol sensitivity. Resolution of the merodiploid was confirmed by PCR amplification.

Statistical analysis. A Kaplan-Meier survival analysis with log rank significance test was performed on the survival curves in the fish infection experiment. Student's T-tests assuming unequal variances were used for experiments containing two data groups. One-way ANOVA with Tukey post hoc test was performed for all other experiments. *P* values of <0.05 were considered statistically significant.

Results

Identification and mutant construction of TCA cycle genes in *V. anguillarum*. In order to identify gene targets for mutagenesis the published genomes of *V. anguillarum* strains 775, 96F, M3, NB10, RV22, 90-11-286 and the *V. anguillarum* M93Sm draft genome (unpublished data) annotated by RAST were examined and found to have the following TCA cycle genes/operons: *gltA, acnB, icd, sucAB, sucCD, sdhCDAB, frdABCD fumA,* and *mdh* (Fig. 1 and Table 3). While this set of genes allows for a fully functional TCA cycle, none of the strains have a *fumC* gene, which encodes the aerobic fumarate class II hydratase. All strains also lack the anaerobic fumarate hydratase (*fumB*) gene. Additionally, all strains possessed *cra*, which encodes the repressor of fructose metabolism in *S. enterica* [22]. The *V. anguillarum* M93Sm sequences for the *icd, sucA, sucC, sdhC, fumA, mdh,* and *cra* genes were used to create insertional mutations in *V. anguillarum* M93Sm. The seven mutant strains and the one restored strain listed in Table 1 were constructed using the primers listed in Table 2 as described in the Methods.

***icd* mutant is highly attenuated for virulence against rainbow trout.** The virulence of the seven *V. anguillarum* metabolism mutants were tested on rainbow trout and compared to wild type M93Sm in order to determine if mutations in metabolism could affect pathogenesis. Groups of five fish were infected by IP-injection (as described in the

Methods) with either the wild type (M93Sm), *icd* mutant (XM420), *sucA* mutant (XM440), *sucC* mutant (XM450), *sdhC* mutant (XM460), *fumA* mutant (XM470), *mdh* mutant (XM410) or *cra* mutant (XM430) in NSS at a dosage of ~2×10^5 CFU per fish. Injection with NSS only served as a negative control (Mock). During the 14-day observation window, 40% of M93Sm infected fish survived. Fish infected with the *sucA* mutant, *sdhC* mutant or *icd* mutant had a higher survival percentage than M93Sm (50% for *sucA* mutant, 80% for *sdhC* mutant, and 100% for *icd* mutant); however, only the difference between the *icd* mutant and M93Sm was statistically significant ($p = 0.037$) (Fig 2A). The experiment was repeated using a two-fold higher dose (~4×10^5 CFU per fish) of M93Sm and the three mutant strains (*icd* mutant, *sucA* mutant and *sdhC* mutant) that exhibited attenuated virulence in the previous experiment. At this dose, only 20% of M93Sm-infected fish survived. Only the *icd* mutant-infected fish had a statistically significant higher survival percentage (100%) compared to M93Sm ($p = 0.0153$) (Fig 2B). The data indicate the *icd* mutant is avirulent in these experimental conditions.

Further, we tested the virulence of M93Sm and the *icd* mutant by another infection route. Groups of 10 fish were infected by immersion as described in the Methods with M93Sm or *icd* mutant in 1.5% salt solution at a dose of ~4×10^6 CFU/ml, or just immersed in a 1.5% salt solution without *V. anguillarum* as a negative control (Mock). During the 14-day observation window, there was a statistically significant difference ($p = 0.007$) between the survival of M93Sm infected fish (30%) and *icd* mutant infected fish (90%) (Fig. 3). Taken together, the IP infection data and the immersion infection data demonstrate that the *icd* mutant is highly attenuated for infection in rainbow trout.

Pre-treatment by immersion with the *icd* mutant protected rainbow trout from the subsequent challenge of *V. anguillarum* M93Sm. Fish previously challenged by immersion with the *icd* mutant were subsequently challenged with the wild type M93Sm strain to test if the *icd* mutant was immunogenic. Six weeks after the initial infection, a group of five fish that survived the initial infection with the *icd* mutant (labeled as "treated with the *icd* mutant" in Fig. 4) and a group of five "untreated" fish were infected via immersion with M93Sm at a dose of ~4×10^6 CFU/ml and were observed for 14 days. By day 2 all fish in the untreated group died. All fish in the group treated with the *icd* mutant survived the 14-day observation period. The difference between the two experimental groups was statistically significant ($p = 0.008$). The results indicate that the *icd* mutant is immunogenic and protective against wild type infection when administered by immersion.

All mutants exhibited either same or higher expression levels of the three hemolysin genes compared to wild type. Vah1, RtxA, and Plp are the three hemolysins found in M93Sm and are responsible for the hemolytic/cytolytic activity against fish erythrocytes, leukocyte and epithelial cells [11, 7, 9] and unpublished data]. We tested the expression of *vah1*, *rtxA* and *plp* during exponential phase to determine whether mutations in metabolism could affect the expression of these hemolysin genes. Data indicate that in all mutants except the *icd* mutant, expression of *vah1* and *plp* were up regulated by 1.49-16.15-fold compared to M93Sm with most of the changes being significant (Fig. 5). In the *icd* mutant, expression of *plp* was up regulated by 1.76-fold while the expression of *vah1* was slightly decreased (to 49% of WT), neither of which was a significant change from M93Sm (Fig. 5). Plp is the most efficient hemolysin against fish erythrocytes [11].

TCA cycle mutants with an increased expression of *plp* also demonstrated an increased zone of hemolysis on 5% fish blood agar plates (Fig. S2). There was no change in the zone of hemolysis for the *icd* mutant. Expression of *rtxA* in all mutants was not significantly different from M93Sm (Fig. 5). Taken together, all metabolism mutants have the same or higher expression levels of hemolysin genes compared to the wild type.

***icd* mutant exhibited significant lower cell density limit than wild type in two forms of rich media.** Fig. 6 shows the typical growth curves for the wild type *V. anguillarum* M93Sm and the seven metabolism mutants in LB20 broth. In these growth conditions, M93Sm, the *icd* mutant, and the *cra* mutant exhibited classic bacterial growth curves with a lag phase, an exponential phase and a stationary phase. The *sucA, sucC, sdhC, fumA* and *mdh* mutants all exhibited a two-stage growth curve, with each stage consisting of a lag phase and an exponential phase. The exponential phase in the first growth stage was named exponential phase I and the exponential phase in the second growth stage was named exponential phase II. The generation times of the exponential phases of all mutants were longer than for M93Sm (Table 4). The final cell density (measured by OD_{600}) of the *icd* mutant after 23 h was the lowest among all strains. Similarly, after 24 h of growth in LB20 the final cell density (CFU/ml) of the *icd* mutant was 47% that of M93Sm (Table 5) and the difference is significant (p = 0.011). M93Sm and the *icd* mutant were grown in NSS supplemented with 200 µg protein/ml of fish gastrointestinal mucus (NSSM) to better replicate conditions within a host. After 24 h of growth in NSSM the final cell density of the *icd* mutant was only ~31% of that for M93Sm (Table 5) and the difference is significant (p = 0.007).

Growth in LB20 supplemented with 118 mM glutamate restores growth of the *icd*

mutant to wild type levels. The *icd* mutant is unable convert isocitrate into α-ketoglutarate, the immediate precursor of glutamate. Consequently, the *icd* mutant was only able to grow in 3M + 0.15% glucose with the addition of glutamate (Fig. 7A). Glutamate was added to LB20 to determine if the *icd* mutant final cell density would increase. Fig. 7B shows the typical growth curves of M93Sm and the *icd* mutant in LB20 with (solid lines) and without (dashed lines) the addition of 118 mM of glutamate. After 24 h, M93Sm and the *icd* mutant grew to similar final cell densities when LB20 was supplemented with 118 mM glutamate. Additionally, Fig. 7C demonstrate that decreasing the amount of glutamate (from 118 mM to 2.95 mM) added to LB20 decreases the final cell density of the *icd* mutant, but not M93Sm, after 24 h of growth. The final cell density of the *icd* mutant was not restored to a wild type level when LB20 was supplemented with glucose, succinate (Fig. S3) or gluconate (Fig. 7C).

Resolving the merodiploid in the *icd* mutant restores growth and pathogenicity. A revertant to the wild type *icd* gene was selected to demonstrate that the *icd* mutant (XM420, a merodiploid with an insertion in the *icd* gene) contained no additional mutations that could be causing the loss of pathogenicity and decreased cell density. Initially, attempts were made to complement the *icd* mutant in *trans* by cloning *icd* and its native promoter into the pSUP203 vector; however, all pSUP203-*icd* vectors isolated from *E. coli* SM10 contained single nucleotide substitutions that resulted in amino acid changes in *icd* that inactivated isocitrate dehydrogenase (data not shown). Since the *icd* mutant is unable to grow on 3M + glucose, *icd* mutants that spontaneously resolved the merodiploid were isolated on 3M + glucose agar plates as described in the Methods. The reversion rate of the *icd* mutant to a wild type phenotype grown in LB20 overnight was

calculated to be 1 out of 1.6×10^{10} cells. Fig. S1 shows the typical growth curves for M93Sm, the *icd* mutant and the restored *icd* strain in LB20 and 3M + 0.15% glucose. M93Sm and the restored *icd* strain were able to grow in 3M + 0.15% glucose unlike the *icd* mutant (Fig S1A). Additionally, when the strains were grown in LB20 the final cell density returned to wild type levels when *icd* was restored (Fig. S1B). To determine if restoring *icd* restores pathogenicity, juvenile rainbow trout were challenged via immersion with M93Sm, the *icd* mutant and the restored *icd* strain at a dose of between 4×10^6 and 8×10^6 CFU/ml. After day 8, 26% (5/19) of the M93Sm challenged fish, 40% (6/15) of the restored *icd* challenged fish and 95% (19/20) of the *icd* mutant challenged fish survived (Fig. 8). There was no statistically significant difference between M93Sm and the restored *icd* strain (P = 0.50). Again, there was a statistically significant difference between M93Sm and the *icd* mutant ($p < 0.00004$). The results indicate that when the merodiploid present in the *icd* mutant is resolved, wild type levels of growth in 3M + 0.15% glucose and LB20 and pathogenicity against juvenile rainbow trout is returned.

Discussion

The tricarboxylic acid (TCA) cycle is involved in the generation of energy through the oxidation of acetate. TCA intermediates serve as precursor metabolites for the synthesis of amino acids and peptidoglycan. The M93Sm genome along with the published genomes of *V. anguillarum* strains 775, 96F, M3, NB10, RV22, 90-11-286 were examined for TCA cycle enzymes and the following genes were found: *gltA, acnB, icd, sucAB, sucCD, sdhCDAB, frdABCD fumA,* and *mdh* (Fig. 1 and Table 3). Additionally, *cra*, which encodes the repressor of fructose metabolism in *S. enterica* and

E. coli and has previously been shown to be essential for *S. enterica* virulence, is present in the *V. anguillarum* genomes [22].

When in a nutrient limited environment, bacteria must be able to synthesize any essential metabolites that are not freely available in order to grow. Previous studies have shown that mutations in central metabolism genes result in attenuation of virulence in several intracellular pathogens including *S. enterica*, uropathogenic *E. coli* (UPEC), *M. tuberculosis* and *E. ictaluri* [19, 21, 26, 23-25, 17, 18]. These observations suggest that central metabolism is necessary for these intracellular pathogens to function inside the nutrient-limited environment of the phagosome; however, *V. anguillarum* is not an intracellular pathogen. While some studies have suggested that *V. anguillarum* can survive internally in fish epithelial cells and CHSE cells (derived from pooled embryonic cells from *Oncorhynchus tshawytscha*), more recent studies have demonstrated that *V. anguillarum* actively evades phagocytosis by fish epithelial cells and cannot survive for 24 h in macrophages [33-36]. In this study, fish were infected with *V. anguillarum* strains by either of two methods: intraperitoneal injection or immersion with both methods resulting in a similar percent survival when fish were challenged with M93Sm (20% for injection, see Fig 2A and 2B; 0%~30% for immersion, see Fig. 3). Only the *icd* mutant had a statistically significant higher level of survival compared to the wild type, 100% for IP injection (Fig 2A and 2B) and 90% for immersion (Fig. 3). It is not thought that reversion of the merodiploid to a wild type phenotype caused the other metabolism mutants to be virulent because Cm resistant colonies were isolated from the organs of dead fish. IP injection bypasses the need for invasion. No mortalities resulted from IP injection with the *icd* mutant indicating that *icd* is required for *V. anguillarum* persistence

and growth in fish tissues. Rainbow trout infected with the *icd* mutant via immersion and subsequently challenged with the M93Sm wild type showed 100% survival (Fig. 4) demonstrating that the *icd* mutant had immunoprotective effects and elicited an adaptive immune response. Moreover, as a proof of concept, the data suggest that an *icd* deletion mutant could be the basis for a live attenuated vaccine against *V. anguillarum* infection.

Our observation that a knockout of the *icd* gene results in attenuation of virulence raises the question of whether expression of required virulence genes is significantly reduced in the mutant and, therefore, results in attenuation. We previously identified and characterized three hemolysin/cytolysin genes and their encoded proteins secreted by *V. anguillarum*: *plp*, *vah1* and *rtxA* [11, 7, 9]. While mutations in *plp* and *vah1* have modest effects on virulence against fish epithelial cells and fish, a knockout mutation in *rtxA* is avirulent in fish [11, 7, 9]. All metabolism mutants exhibited no significant declines in the expression of three hemolysins (Fig. 5) and most of the mutants exhibited increased expression. Accordingly, the *icd* mutant is not attenuated by the lack of hemolysin production because the decrease in *rtxA* and *vah1* expression was not significant; however, future studies could examine the expression of other virulence factors. It is unclear why expression of *plp* and *vah1* is increased in the metabolism mutants. Minato *et al* [37] demonstrated the accumulation of acetyl-CoA in *Vibrio cholerae* central metabolism mutants resulted in an increased expression of its virulence gene activator ToxT. It is possible that accumulation or depletion of certain metabolites in *V. anguillarum* could increase hemolysin/cytolysin expression. Expression of *hlyU,* the positive regulator of the both the *vah1 plp* gene cluster and the *rtxA* gene cluster, was examined and shown to be up-regulated in the *sucA* and *mdh* mutants (data not shown)

[38]. However, the increased expression of *hlyU* may not be the sole explanation for the increased expression of *plp* and *vah1* because an increase in expression of *rtxA* should have also occurred.

The growth rate and final cell density was determined for all metabolism mutants grown in LB20 for 24 h. The slowest growing mutant, *fumA*, was as virulent as the wild type while the mutant with the lowest final cell density, *icd*, was attenuated suggesting that decreased final cell density results in a loss of pathogenicity against rainbow trout (Fig. 2, Fig. 6, Table 4 and Table 5). When the mutation in *icd* was resolved, the restored *icd* strain demonstrated the wild type phenotype for both growth and pathogenicity (Fig. S1A and B and Fig. 8). While it is possible that the insertional mutation affected the expression of the two genes flanking *icd* (ribosomal large subunit pseudouridine synthase E (Accession number: WP_017043910.1) and cold shock domain protein CspD (Accession number: WP_013857087.1)), it is unlikely as neither gene is part of an operon that includes *icd*. Since isocitrate dehydrogenase catalyzes the formation of α-ketoglutarate (the immediate precursor of glutamate) from isocitrate, the *icd* mutant is auxotrophic for glutamate (Fig. 7A). Our data demonstrate that the *icd* mutant stops growing once exogenous glutamate or its derivatives are exhausted (Fig. 7B and Fig. 7C). The data also demonstrate the decreased growth yield was not do to a reduction of ATP production as addition of gluconate or succinate did not restore growth to a wild type cell density. It is interesting that the only other auxotrophic mutant, *sucA*, grows to a wild type cell density in LB20 and is as virulent as the wild type considering it cannot synthesize succinyl-CoA, a metabolite needed for the synthesis of lysine, methionine and diaminopimelic acid. Presumably, succinyl-CoA or its derivatives are not limiting in

LB20 or in fish tissues. Furthermore, this also suggests that the *icd* mutant is primarily starved for glutamate and would not need to synthesize succinyl-CoA by metabolizing glutamate to α-ketoglutarate. We hypothesize that during infection the *icd* mutant is unable to obtain enough α-ketoglutarate derivatives to grow to a wild type cell density and, therefore, cannot reach a cell density necessary for a successful systemic infection. In support, it has previously been demonstrated that a *V. anguillarum* M93Sm *mugA* mutant that was unable to grow in salmon intestinal mucus was avirulent against Atlantic salmon [39]. Additionally, when *V. anguillarum* 775 was cured of its plasmid-encoded siderophore, the mutant was unable to sequester iron and exhibited decreased virulence. [40, 41].

M93Sm is an O2α serotype and the presumed infection route is through the gastrointestinal tract as no necrotic skin lesions have ever been observed with this strain (unpublished data). The *in vitro* growth experiment (Table 5) suggests that there are not enough α-ketoglutarate derivatives in intestinal mucus to support the growth of the *icd* mutant to a wild type cell density even though it is the metabolite with the second highest concentration (3.03 mM) in rainbow trout mucus [42]. It should be noted that for *in vitro* growth experiments the concentrations of glutamate and glutamine in the mucus are not known and the growth conditions represent an ideal environment for growth; *V. anguillarum* does not have to evade the fish immune system or compete with commensal bacteria and it is not expected that the *icd* mutant will grow to the cell density shown in the *in vitro* growth experiments in the fish. As demonstrated by Muroga *et al*, [43] *V. anguillarum* found in the spleen and intestine of moribund fish challenged via immersion only reached a cell density of 4.0×10^8 CFU/g and 2.5×10^7 CFU/g respectively. Altinok *et*

al [44] showed a *V. anguillarum* succinate dehydrogenase mutant was avirulent against rainbow trout when injected at a dose at 10^5 CFU. Similar to our results, the authors showed that the succinate dehydrogenase mutant grew to a cell density slightly lower than the wild type at 12 h; however, the authors failed to show the growth yield at 24 h. Further, the authors did not create a complement strain to demonstrate that the loss of virulence was solely do to mutating *sdhB*. Most importantly, the ATCC has redesignated their strain as a *Pseudomonas* species.

Conclusions

Seven *V. anguillarum* metabolism mutants were created and examined for pathogenicity against juvenile rainbow trout, hemolysin/cytolysin expression and growth in rich media. Of the central metabolism mutants, only the *icd* mutant showed strong attenuation in virulence, which did not result from a decrease in virulence factor expression. In addition, only the *icd* mutant had a final cell density that was lower than the wild type, which resulted from the inability to synthesize α-ketoglutarate and downstream metabolites. Taken together, the data suggest that during infection, if *V. anguillarum* is unable to synthesize essential molecules (e.g. α-ketoglutarate/2-oxoglutarate) and when those molecules or their derivatives (e.g. glutamate, glutamine) become limiting in the host, *V. anguillarum* will be unable to grow to a density necessary to sustain a systemic infection of the host.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
V. anguillarum strains		
M93Sm	Spontaneous Sm^r mutant of M93 (serotype O2a)	[45]
XM420	Sm^r Cm^r; *icd* insertional mutant	This study
ES422	Sm^r; Restored *icd* strain	This study
XM440	Sm^r Cm^r; *sucA* insertional mutant	This study
XM450	Sm^r Cm^r; *sucC* insertional mutant	This study
XM460	Sm^r Cm^r; *sdhC* insertional mutant	This study
XM470	Sm^r Cm^r; *fumA* insertional mutant	This study
XM410	Sm^r Cm^r; *mdh* insertional mutant	This study
XM430	Sm^r Cm^r; *cra* insertional mutant	This study
E. coli strains		
Sm10	*thi thr leu tonA lacY supE recA* RP4-2-Tc::Mu::Km (λ *pir*)	[46]
S100	Km^r; Sm10 containing plasmid pNQ705-1	[47]
Q420	Km^r Cm^r; Sm10 containing plasmid pNQ705-*icd*	This study
Q440	Km^r Cm^r; Sm10 containing plasmid pNQ705-*sucA*	This study
Q450	Km^r Cm^r; Sm10 containing plasmid pNQ705-*sucC*	This study
Q460	Km^r Cm^r; Sm10 containing plasmid pNQ705-*sdhC*	This study
Q470	Km^r Cm^r; Sm10 containing plasmid pNQ705-*fumA*	This study
Q410	Km^r Cm^r; Sm10 containing plasmid pNQ705-*mdh*	This study
Q430	Km^r Cm^r; Sm10 containing plasmid pNQ705-*cra*	This study
Plasmid		
pNQ705-1	Cm^r; suicide vector with R6K origin	[47]
pNQ705-*icd*	Cm^r; For *icd* insertional mutant	This study
pNQ705-*sucA*	Cm^r; For *sucA* insertional mutant	This study
pNQ705-*sucC*	Cm^r; For *sucC* insertional mutant	This study
pNQ705-*sdhC*	Cm^r; For *sdhC* insertional mutant	This study
pNQ705-*fumA*	Cm^r; For *fumA* insertional mutant	This study
pNQ705-*mdh*	Cm^r; For *mdh* insertional mutant	This study
pNQ705-*cra*	Cm^r; For *cra* insertional mutant	This study

Table 2. Primers used in this study.

Primer	Sequence (5' to 3', underlined sequences are designed restriction sites)	Description	Reference
pr31	GGTGAGCTCTATTCTTTATTGCCGATTATC	For *icd* insertional mutant, forward, *Sac*I	This study
pr32	AAATCTAGAGTAAGTCGCTTTAATCGCTTC	For *icd* insertional mutant, reverse, *Xba*I	This study
pr50	AAAGAGCTCGTGATCCAGATGTCGATGCTA	For *sucA* insertional mutant, forward, *Sac*I	This study
pr51	GGTTCTAGAGTTCAGTGTCGATAATGTGCA	For *sucA* insertional mutant, reverse, *Xba*I	This study
pr52	AAAGAGCTCGGTCGGATTAGTACAGCGAAG	For *sucC* insertional mutant, forward, *Sac*I	This study
pr53	GGTTCTAGACTTTTTCAATTTCCACGCCGC	For *sucC* insertional mutant, reverse, *Xba*I	This study
pr54	AAAGAGCTCATGTTCGTTGCGGTCGGAATT	For *sdhC* insertional mutant, forward, *Sac*I	This study
pr55	GGTTCTAGATCCAACTCTTCAAAGTGGCCC	For *sdhC* insertional mutant, reverse, *Xba*I	This study
pr56	GGTGAGCTCTCCTTGCACCATATTGATATG	For *fumA* insertional mutant, forward, *Sac*I	This study
pr57	GGGTCTAGAAGGCTTATCATCGAGAAGAGAG	For *fumA* insertional mutant, reverse, *Xba*I	This study
pr29	GGTGAGCTCATGCCAGCGTTAACATTAAAC	For *mdh* insertional mutant, forward, *Sac*I	This study
pr30	AAATCTAGAGCTGTATGACATCGCACCGGT	For *mdh* insertional mutant, reverse, *Xba*I	This study
pr33	AAAGAGCTCGCGGCGTGAGACTAAGGCATC	For *cra* insertional mutant, forward, *Sac*I	This study
pr34	AAATCTAGACAATGGCAAAGCGCAGAAGTA	For *cra* insertional mutant, reverse, *Xba*I	This study
vah1 F RT	GTTTGGTATGGAACACCGCTCAAG	For *vah1* qRT-PCR, forward	This study
vah1 R RT	GGCTCAACCTCTCCTTGTAACCAA	For *vah1* qRT-PCR, reverse	This study
plp F RT	CAGACGACCACCAGTAACCACTAA	For *plp* qRT-PCR, forward	[8]
plp R RT	GCAATCATGATGACCCAGCAACAG	For *plp* qRT-PCR, reverse	[8]
Pm111	GGAAATTATTCCGCCGACGATGGA	For *rtxA* qRT-PCR, forward	[7]
Pm112	GCCGATACCGTATCGTTACCTGAA	For *rtxA* qRT-PCR, reverse	[7]

Table 3. Metabolism genes examined in this study

Gene or operon	Product	Present in sequenced *V. anguillarum* strains [a]
gltA	Type II citrate synthase	Yes
acnB	Aconitate hydratase B	Yes
icd	Isocitrate dehydrogenase	Yes
sucAB	2-oxoglutarate dehydrogenase (E1 component, E2 component)	Yes
sucCD	Succinyl-CoA synthetase (beta subunit, alpha subunit)	Yes
sdhCDAB	Succinate dehydrogenase (cytochrome b556 subunit, membrane anchor subunit, flavoprotein subunit, iron-sulfur protein)	Yes
frdABCD	Fumarate reductase (flavoprotein subunit, iron-sulfur subunit, anchor subunit, anchor subunit)	Yes
fumAC	Aerobic fumarate hydratase (class I, class II)	*fumA*: Yes; *fumC*: not found
fumB	Anaerobic fumarate hydratase (class I)	Not found
mdh	Malate dehydrogenase	Yes
cra	Fructose repressor protein	Yes

[a] *V. anguillarum* strains: M93Sm 775, 96F, M3, NB10, RV22, 90-11-286

Table 4. Generation times of various *V. anguillarum* strains grown in LB20[1]

Strain	Exponential Phase I (Minutes)	Exponential Phase II (Minutes)
M93Sm	44.00	NA
icd	54.95	NA
sucA	64.32	98.52
sucC	52.42	99.57
sdhC	61.19	101.70
fumA	73.55	89.38
mdh	67.11	115.28
cra	58.59	NA

[1]Values calculated from data presented in Figure 6 during exponential growth.
NA: not applicable

Table 5. Final cell density (CFU/ml) of various *V. anguillarum* cultures grown for 24 h

Strain	CFU/ml in LB20	CFU/ml in NSSM (200 µg/ml)
M93Sm	3.4×10^{9} ($\pm0.3\times10^{9}$)	4.2×10^{9} ($\pm0.7\times10^{9}$)
icd	1.6×10^{9} ($\pm0.02\times10^{9}$)*	1.3×10^{9}($\pm0.3\times10^{9}$)*

*Statistically significant difference compared to M93Sm (p <0.05).

Figure Legends

Figure 1. Embden-Meyerhoff-Parnas Pathway, TCA cycle, and metabolism of fructose. The arrows indicate the physiological directions of the reactions. The gene symbols of the enzyme for each reaction are listed beside the reaction. Boxed genes indicate the genes that were mutated in this study (Table 1).

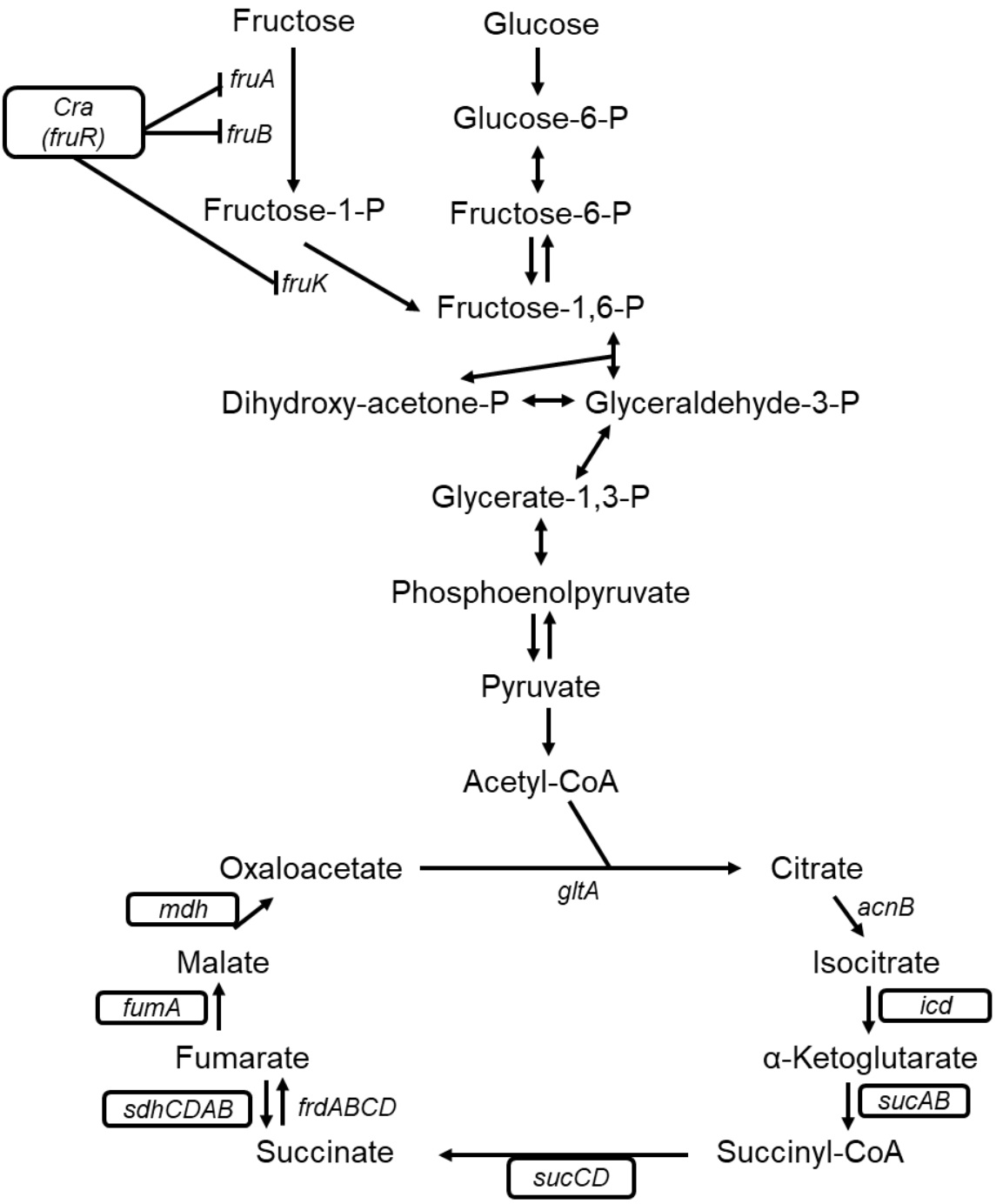
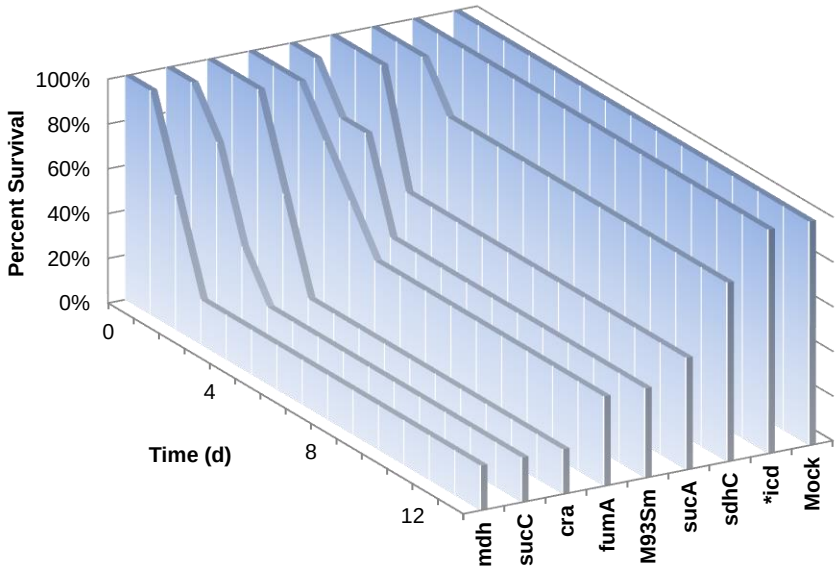

Fructose
Glucose
fruA
Cra
(fruR)
fruB
Glucose-6-P
Fructose-1-P
Fructose-6-P
fruK
Fructose-1,6-P
Dihydroxy-acetone-P
Glyceraldehyde-3-P
Glycerate-1,3-P
Phosphoenolpyruvate
Pyruvate
Acetyl-CoA
Oxaloacetate
gltA
Citrate
mdh
acnB
Malate
Isocitrate
fumA
icd
Fumarate
α-Ketoglutarate
sdhCDAB
frdABCD
sucAB
Succinate
Succinyl-CoA
sucCD

Figure 2. Percent survival of rainbow trout IP injected with *V. anguillarum* wild type (M93Sm) and various mutant strains at a dosage of **A)** 2×10^5 CFU/fish and **B)** 4×10^5 CFU/fish. Negative control groups of fish (Mock) were injected with sterile NSS. Five fish were used for each treatment. (One fish treated with the *sucA* mutant died, but not from vibriosis and no *V. anguillarum* were recovered, so only four fish were counted). *Statistically significant difference compared to M93Sm (p <0.05).

A


Percent Survival
100%
80%
60%
40%
20%
0%
0
4
8
12
Time (d)
mdh
sucC
cra
fumA
M93Sm
sucA
sdhC
*icd
Mock

B

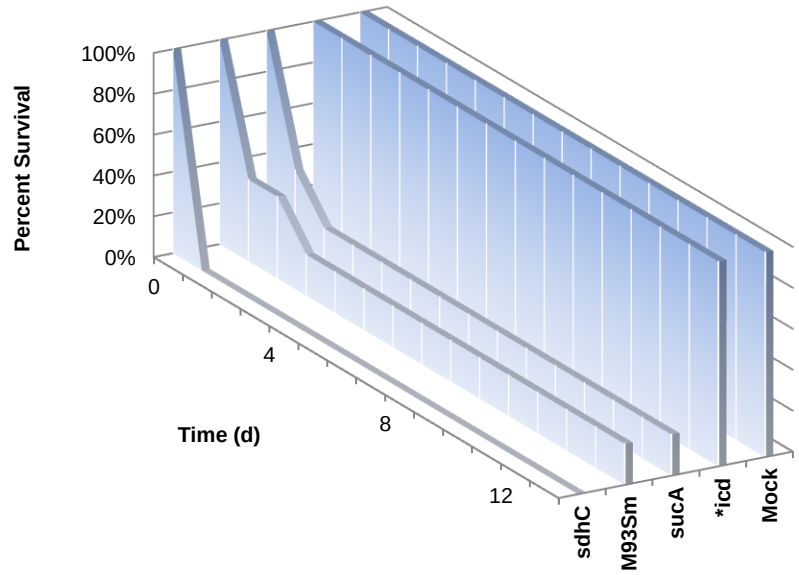

Percent Survival
100%
80%
60%
40%
20%
0%
0
4
8
12
Time (d)
sdhC
M93Sm
sucA
*icd
Mock

Figure 3. Percent survival of rainbow trout infected by immersion with *V. anguillarum* strains M93Sm (wild type) or XM420 (*icd*) at a dose of 4×10^6 CFU/ml. A negative control group of fish (Mock) were immersed in sterile NSS. Ten fish were used for each treatment. *Statistically significant difference compared to M93Sm (p <0.05).

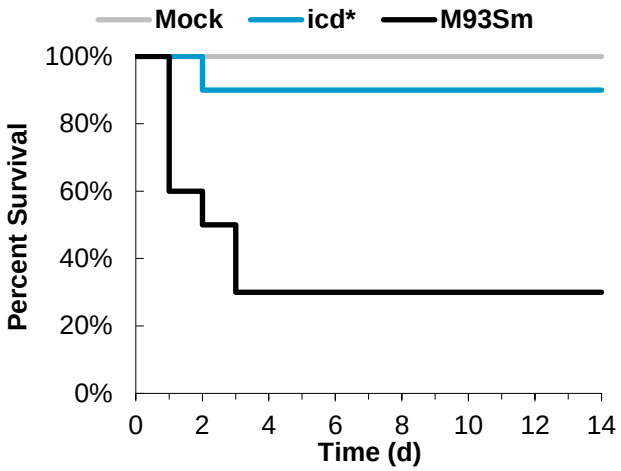

Mock
icd*
M93Sm
100%
80%
60%
40%
20%
0%
Percent Survival
0
2
4
6
8
10
12
14
Time (d)

Figure 4. Percent survival of immersion vaccinated rainbow trout. Rainbow trout were sham vaccinated with NSS (labeled as "untreated") or immersed vaccinated with the *icd* mutant (Labeled as "treated with *icd*") and challenged with wild type *V. anguillarum* M93Sm (4×10^6 CFU/ml). Five fish were used for each treatment. *Statistically significant difference compared to M93Sm ($p < 0.05$).

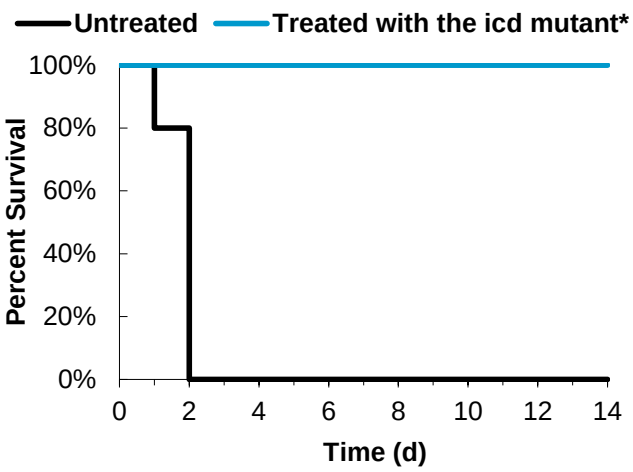

Untreated
Treated with the icd mutant*
100%
80%
60%
40%
20%
0%
Percent Survival
0
2
4
6
8
10
12
14
Time (d)

Figure 5. Relative expression of *vah1*, *plp*, *rtxA* determined by qRT-PCR analysis of *V. anguillarum* wild-type (M93Sm) and various TCA mutants during logarithmic (Log)-phase growth. The data presented are representative of two independent experiments. Each value is the average for three replicates. Between marked strains and M93Sm: * $p < 0.05$ and *** $p < 0.001$. Error bars represent 1 standard deviation.

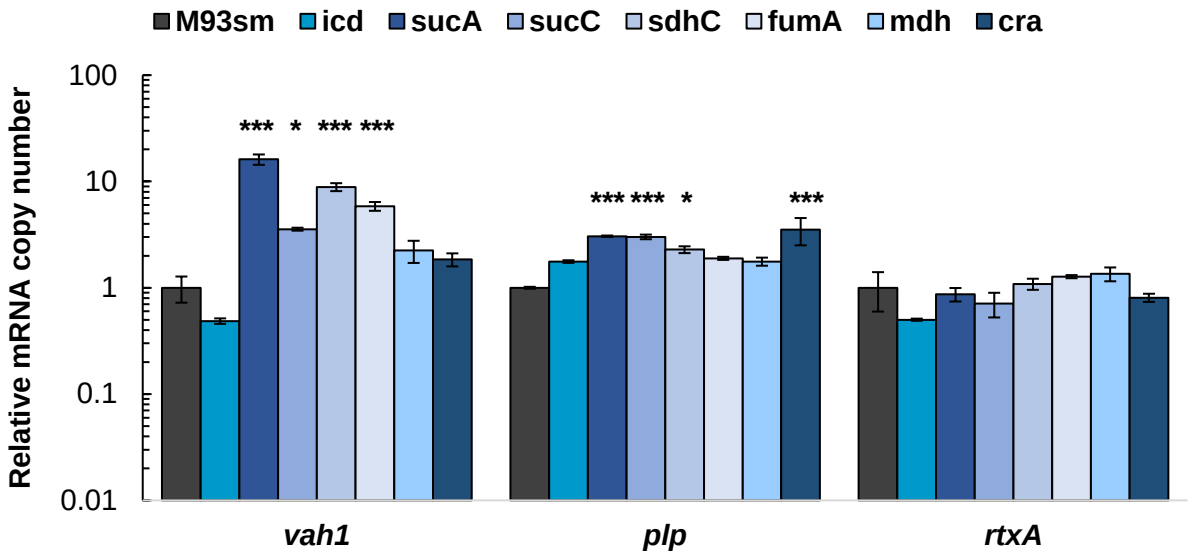

M93sm
icd
sucA
sucC
sdhC
fumA
mdh
cra
Relative mRNA copy number
100
10
1
0.1
0.01
*** * *** ***
*** *** * ***
vah1
plp
rtxA

Figure 6. Growth curves of various *V. anguillarum* strains grown in LB20 at 27°C with shaking (200 rpm). At various time points after inoculation samples were taken for determination of optical density at 600 nm (OD_{600}). The data are from one representative experiment.

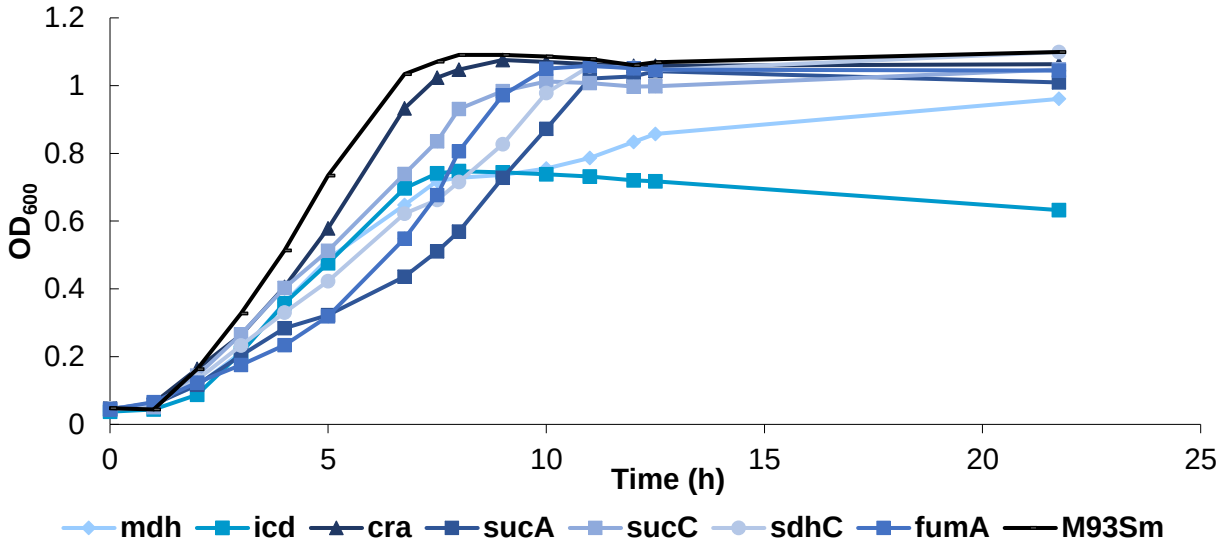
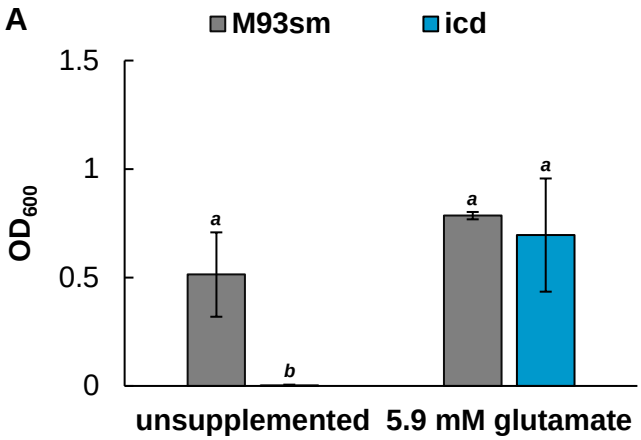
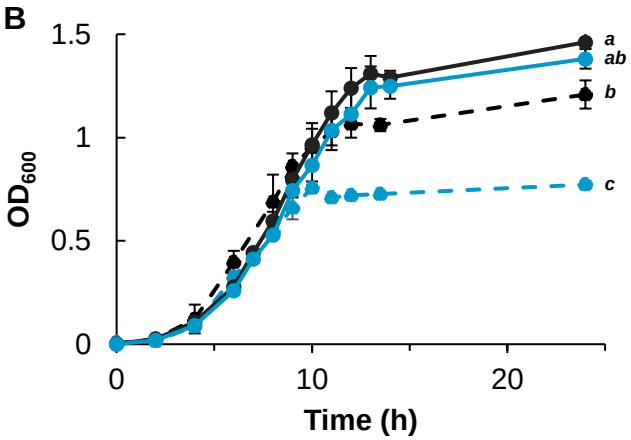

1.2
1
0.8
0.6
0.4
0.2
0
OD600
0
5
10
15
20
25
Time (h)
mdh
icd
cra
sucA
sucC
sdhC
fumA
M93Sm

Figure 7. Growth of *V. anguillarum* WT (M93Sm) and the *icd* mutant under various conditions. **A)** Final cell densities (OD_{600}) of *V. anguillarum* strains after 24 h of growth in 3M plus 0.15% glucose supplemented with or without 5.9 mM glutamate. **B)** Growth curves of *V. anguillarum* M93Sm (black) and the *icd* mutant (blue) in LB20 (dashed lines) or LB20 supplemented with 118 mM glutamate (solid lines). Statistical analysis was based on data at 24 h. **C)** Final cell densities (OD_{600}) of *V. anguillarum* M93Sm and *icd* mutant strains grown in LB20 supplemented with decreasing amounts of glutamate. In each experiment cells grown overnight in LB20 were washed in NSS and used to inoculate the appropriate media. Cultures were incubated at 27^{o}C in a shaking water bath (200 rpm) and at various time points after inoculation samples were taken for determination of optical density at 600 nm (OD_{600}). Different letters indicate statistical significance among groups ($p < 0.05$). Error bars represent 1 standard deviation.


A
M93sm
icd
1.5
1
0.5
0
OD600
a
b
a
a
unsupplemented
5.9 mM glutamate


B
1.5
1
0.5
0
OD600
0
10
20
Time (h)
a
ab
b
c

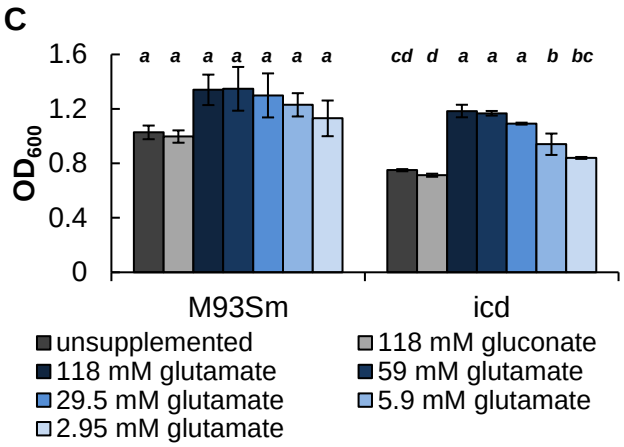
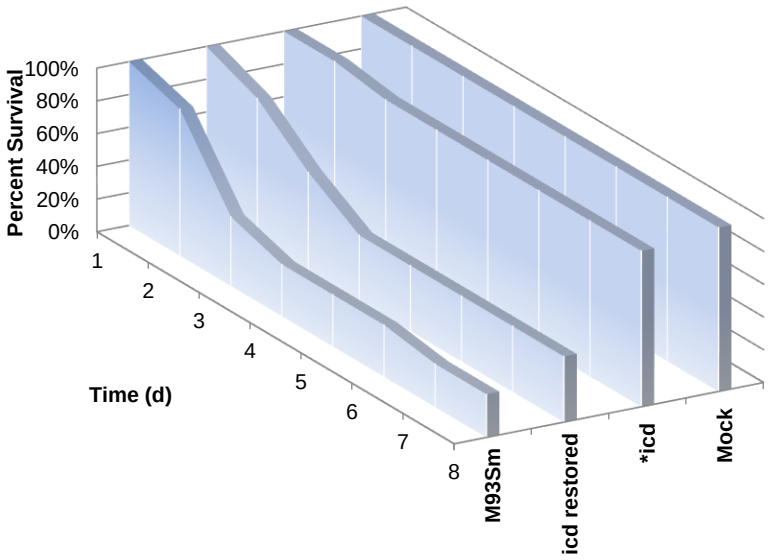
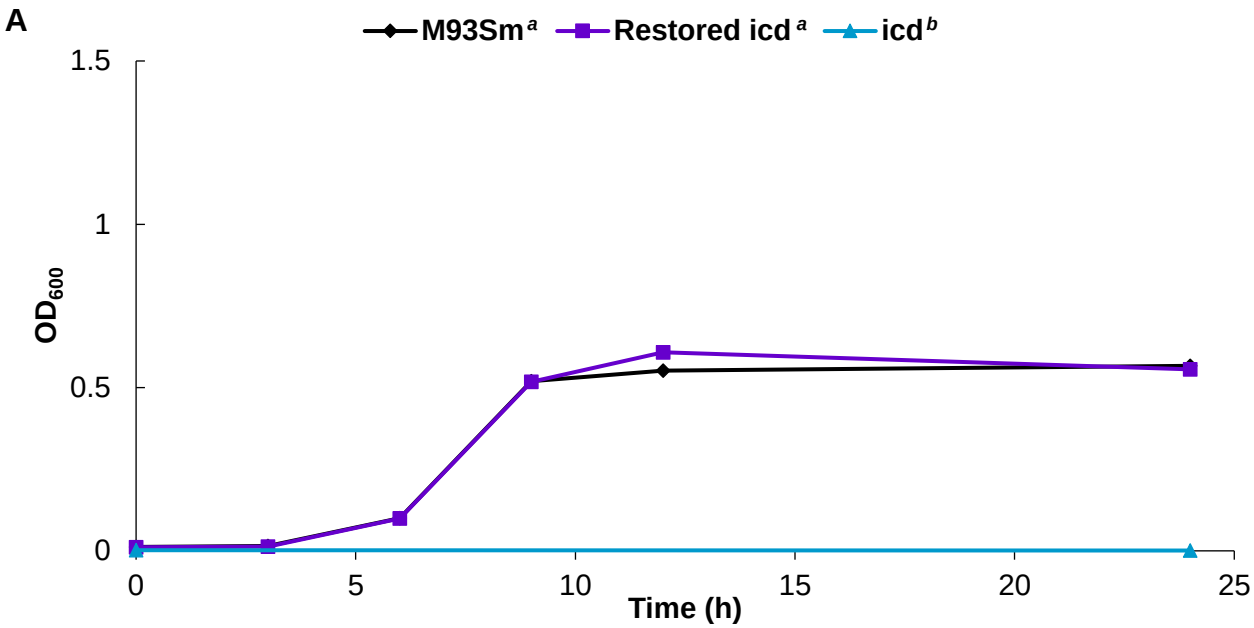
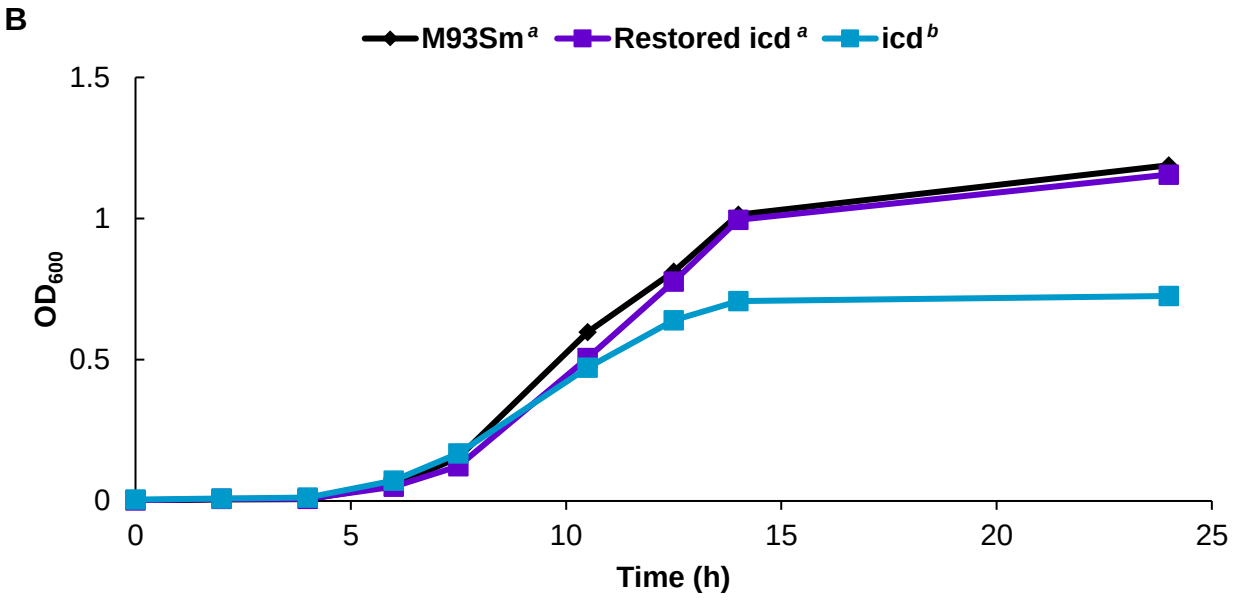

C
1.6
1.2
0.8
0.4
0
OD600
a a a a a a a
cd d a a a b bc
M93Sm
icd
unsupplemented
118 mM gluconate
118 mM glutamate
59 mM glutamate
29.5 mM glutamate
5.9 mM glutamate
2.95 mM glutamate

Figure 8. Percent survival of rainbow trout immersed with various *V. anguillarum* strains at a dosage of 4×10^6 to 7×10^6 CFU/ml. Five fish were used for the uninfected (mock) group. Fifteen fish were treated with the restored *icd* strain. Nineteen fish were treated with M93Sm and twenty fish were treated with the *icd* mutant. *Statistically significant difference compared to M93Sm ($p < 0.01$).

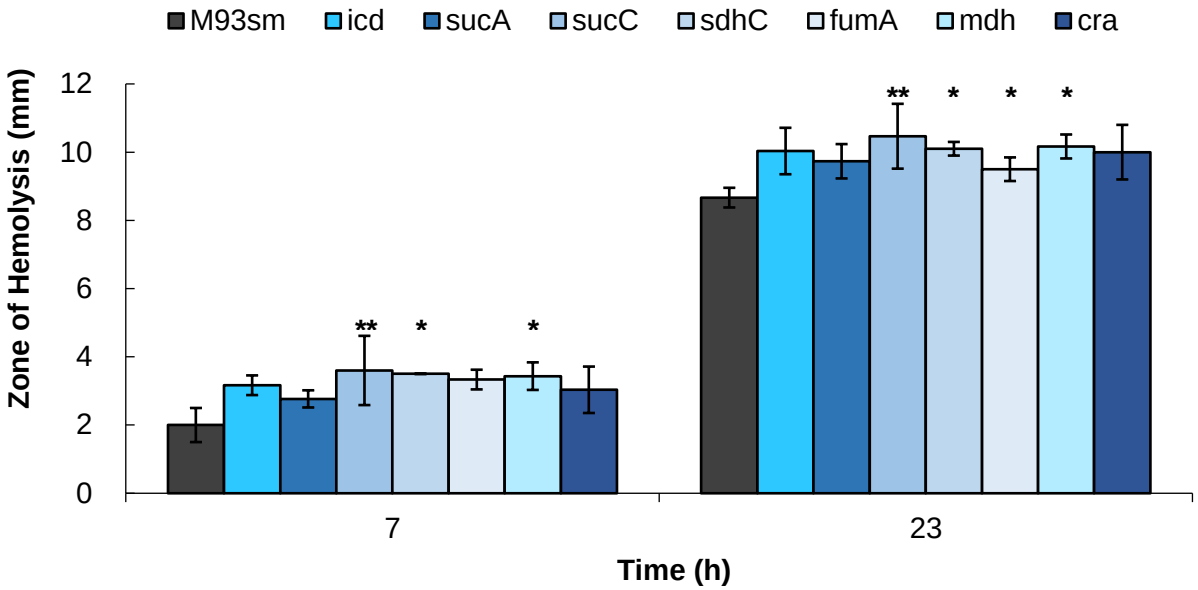

Percent Survival
100%
80%
60%
40%
20%
0%
1
2
3
4
5
6
7
8
Time (d)
M93Sm
icd restored
*icd
Mock

Supplemental Figure 1. Growth curves of *V. anguillarum* strains M93Sm (WT), the *icd* mutant and the restored *icd* strain grown in **A)** 3M + 0.15% glucose and **B)** LB20. In each experiment cells grown overnight in LB20 at 27°C were washed in NSS and used to inoculate the appropriate media. Cultures were incubated at 27°C in a shaking water bath (200 rpm) and at various times after inoculation, samples were taken for determination of optical density at 600 nm (OD_{600}). Different letters indicate statistical significance among groups ($p < 0.05$). Statistical analysis was based on data of stationary phase (>12 h).

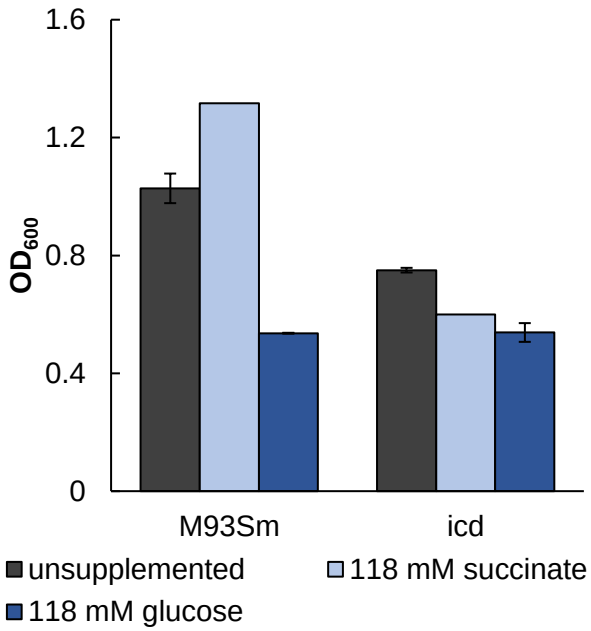

A
M93Sm a
Restored icd a
icd b
1.5
1
0.5
0
OD600
0
5
10
15
20
25
Time (h)


B
M93Sm a
Restored icd a
icd b
1.5
1
0.5
0
OD600
0
5
10
15
20
25
Time (h)

Supplemental Figure 2. Hemolytic activity of various *V. anguillarum* strains grown on fish blood agar. Colonies grown overnight on LB20 plates were tooth picked onto LB20 + 5% trout blood agar plates. The diameter of the zones of hemolysis were measured after 7 h and 23 h of growth at 27°C. Between marked strains and M93Sm: * $p < 0.05$ and ** $p < 0.01$. Error bars represent 1 standard deviation.


M93sm
icd
sucA
sucC
sdhC
fumA
mdh
cra
Zone of Hemolysis (mm)
12
10
8
6
4
2
0
**
*
*
**
*
*
*
7
23
Time (h)

Supplemental Figure 3. Final cell densities (OD_{600}) of *V. anguillarum* WT (M93Sm) and the *icd* mutant after 24 h of growth in LB20 supplemented with or without 118 mM glucose and 118 mM succinate. Error bars represent 1 standard deviation.


1.6
1.2
0.8
0.4
0
OD600
M93Sm
icd
unsupplemented
118 mM succinate
118 mM glucose

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Manuscript-II

Prepare for submission to BMC Microbiology

Characterization of the growth and virulence of a *Vibrio anguillarum* citrate synthase mutant

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Abstract

Background: *Vibrio anguillarum* is an extracellular bacterial pathogen that is a causative agent of vibriosis in finfish and crustaceans with mortality rates ranging from 30% to 100%. Previously, a *V. anguillarum* M93Sm isocitrate dehydrogenase mutant auxotrophic for glutamate was demonstrated to be attenuated in virulence against juvenile rainbow trout. The inability of the mutant to synthesize essential metabolites (i.e. α-ketoglutarate and derivatives) was hypothesized to cause the attenuation. In this study, a citrate synthase mutant was created and characterized to determine if another mutant that is auxotrophic for glutamate would be attenuated.

Results: A citrate synthase (*gltA)* deletion mutant was created and characterized with regard to growth in minimal and complex media, *in vitro* expression of virulence genes, and virulence in juvenile rainbow trout (*Oncorhynchus mykiss*). The Δ*gltA* mutant exhibited a decreased final cell density that resulted from the exhaustion of glutamate from the media. There was no significant decrease in the expression of the three hemolysin genes when detected by qRT-PCR or mortality during infection experiments. A Δ*gltA* mutant capable of growing in minimal media was isolated and shown to have a spontaneous mutation in the transcriptional activator of 2-methylcitrate synthase (*prpR*). This mutation resulted in an increase in expression of 2-methylcitrate synthase (*prpC*). This Δ*gltA prpR*(R66L) mutant exhibited a growth advantage compared to the Δ*gltA* mutant after 24 h in spleen extract medium. Further, after growing 120 h in spleen extract medium, colonies of Δ*gltA* mutants were shown to be capable of growing in minimal media. Δ*prpC* and a Δ*gltA* Δ*prpC* mutants were created and characterized with regard to growth in minimal and complex media and virulence in juvenile rainbow trout. The Δ*gltA*

Δ*prpC* mutant had no growth advantage in spleen extract medium compared to the Δ*gltA* mutant in spleen extract medium but unexpectedly, was still as virulent as the wild type against rainbow trout. The Δ*prpC* mutant was similar to the wild type in regards to both growth in minimal and complex media and virulence against rainbow trout.

Conclusions: The data strongly suggests that simple starvation for glutamate will not directly result in attenuation of virulence. Additionally, spontaneous mutations can occur that compensate for the original gene deletion if the new mutation can replace or bypass the lost metabolic reaction and results in a growth advantage.

Keywords: *Vibrio anguillarum*, Central metabolism, TCA cycle, vibriosis, citrate synthase, virulence

Background

Vibrio anguillarum is a causative agent of warm water vibriosis in finfish, crustaceans and bivalves. Morality rates from infection reach between 30% to 100% and result in severe economic losses to aquaculture industries worldwide [1, 2]. Typically, systemic infection causes fish to die within 1 to 4 days [3-6].

V. anguillarum is an extracellular pathogen capable of invading its host through the gills, skin and intestines [7, 8]. Numerous virulence factors that have been shown to be important during infection include extracellular proteases, hemolytic cytotoxins, iron acquisition systems (siderophores), lipopolysaccharides, chemotaxis, and flagella [9]. Three secreted hemolytic cytotoxins, exhibiting activity against multiple cell types have been characterized in *V. anguillarum*: Vah1 (HlyA homolog, encoded by *vah1*), phospholipase Plp (a phosphatidylcholine-specific phospholipase, encoded by *plp*), and the MARTX toxin RtxA (encoded by *rtxA*) [4, 6, 10]. Expression of the cytotoxins is

under control of the transcriptional activator HlyU and the repressor H-NS [5, 11]. Mutations in *vah1* and/or *plp* resulted in a slight attenuation in virulence against juvenile Atlantic salmon (*Salmo salar*); however, *rtxA* mutants cannot persist in host tissues and are avirulent against juvenile Atlantic salmon challenged via intraperitoneal injection [4, 6, 10].

Several bacterial species that are auxotrophic for aromatic compounds have been shown to be avirulent [12-16]. More recently, central metabolism mutants have been shown to be attenuated in pathogenicity in the intracellular pathogens *Salmonella enterica*, uropathogenic *Escherichia coli* (UPEC), *Mycobacterium tuberculosis*, and *Edwardsiella ictaluri*, [17-27]. The specific gene mutations that cause attenuation in virulence are hypothesized to reflect the metabolic reactions necessary to grow in the nutrient limited environment of the phagosome; if the pathogens cannot utilize the available nutrients they will fail to multiply to the threshold needed to cause disease [18, 26]. Recently, a *V. anguillarum* M93Sm *icd* mutant was shown to be highly attenuated and immunoprotective in juvenile rainbow trout (*Oncorhynchus mykiss*) [28]. *In vitro* growth experiments demonstrated that the *icd* mutant grew to a lower cell density (~60%) than the wild type in two forms of rich media, due to limiting amounts of α-ketoglutarate derivatives (glutamate and glutamine) in the media. In this study, another TCA cycle mutant auxotrophic for glutamate was constructed by deleting the citrate synthase gene (*gltA*). The Δ*gltA* mutant was characterized for growth, hemolysin expression, and pathogenicity against juvenile rainbow trout. Additionally, a Δ*gltA* mutant strain containing a spontaneous mutation in the transcriptional regulator *prpR* capable of growing in 3M + 0.15% glucose (a medium which should be unable to support the

growth of Δ*gltA* mutant) was isolated and characterized for growth in multiple forms of rich media composed of fish extracts that represent different stages of the infection process. Consequently, a Δ*prpC* (2-methylcitrate synthase) and a Δ*gltA* Δ*prpC* mutant were created and characterized for growth in multiple forms of media and pathogenicity against juvenile rainbow trout.

Methods

Bacterial strains, plasmids, and growth conditions. *V. anguillarum* strains (Table 1) were routinely grown in Lysogeny broth containing 2% NaCl (LB20) [29], LB20 + 118 mM glutamate, Marine Minimum Median (3M) + 0.15% glucose [30], NSSM (NSS supplemented with 200μg/ml of fish intestinal mucus) or Spleen extract medium supplemented with the appropriate antibiotic, in a shaking water bath at 27°C. Spleens, extracted from previously euthanized rainbow or brook trout, were added to 1 ml of NSS, placed in an ice-bath and sonicated using Fisher Scientific Sonic Dismembrator Model 500 four times at 10% power for a continuous 10 s. Tubes were then centrifuged (6000 × *g*, 10 min) and the supernatant was collected and centrifuged again (9000 × *g*, 10 min). 1 ml aliquots of the supernatant were added to a sterile 6-well plate (CytoOne) and sterilized with ultraviolet light using a Hoefer UV500 (Time = 10 min). An aliquot of the supernatant was added to LB20 and observed for growth to ensure that the spleen extract was sterile. *E. coli* strains (Table 1) were routinely grown in Lysogeny broth containing 1% NaCl (LB10) supplemented with the appropriate antibiotic, in a shaking water bath at 37°C. Antibiotics were used at the following concentrations: streptomycin, 200 μg/ml (Sm^{200}); chloramphenicol, 20 μg/ml (Cm^{20}) for *E. coli* and 5 μg/ml (Cm^{5}) for *V. anguillarum*.

Allelic exchange mutagenesis. Deletion mutations were made by using a modification of the procedure described by Milton *et al.* [31]. Genes of interested were identified using the M93Sm genome (Accession Number: NOWD00000000) previously annotated by RAST [33]. Briefly, primers (Table 2) were designed based on the target gene sequence of M93Sm. A 250 or 401 bp DNA fragment of the 5' region and the 3' region of the target gene was PCR amplified and ligated using the Gibson assembly method into the suicide vector pDM4 (Table 1) previously digested with SphI or SacI [32]. The Gibson assembly mixture was introduced into *E. coli* SM10 by electroporation using a BioRad Gene Pulser II (BioRad, Hercules, CA). Transformants were selected on LB10 Cm^{20} agar plates. The construction of the recombinant pDM4 was confirmed by PCR amplification. The mobilizable suicide vector was transferred from *E. coli* SM10 into *V. anguillarum* by conjugation. Single-crossover transconjugants were selected with LB20 Sm^{200} Cm^{5} plates, and subsequently, double-crossover transconjugants were selected with LB20 Sm^{200} plates containing 5% sucrose. The resulting *V. anguillarum* mutants were checked for the desired allelic exchange by PCR amplification using primers (Table 2) flanking the deletion.

Cell growth experiments. To cultivate cells for growth experiments, *V. anguillarum* cells grown overnight at 27°C in LB20 supplemented with the appropriate antibiotics were harvested by centrifugation (9,000 × *g*, 2 min), washed twice and resuspended in in NSS. A 200 µl aliquot of the *V. anguillarum* NSS suspension was transferred into a 96-well plate with a clear flat bottom and the optical density at 600 nm (OD_{600}) was read by a VersaMax™ Absorbance Microplate Reader (Molecular Devices). The *V. anguillarum* NSS suspension was prepared to an OD_{600} between 0.400 and 0.500

(~4 × 10^7 CFU/ml) and diluted 1:100 into fresh media. Growth was monitored either by measurement of the OD_{600} or by serial dilution and plate counts.

RNA isolation. Exponential phase cells (3.9×10^7 to 7.3×10^7 CFU/ml) of various *V. anguillarum* strains were treated with RNAprotect Bacteria Reagent (QIAGEN) following the manufacturer's instructions. Total RNA was isolated using the RNeasy kit following the instructions of the manufacturer. All purified RNA samples were quantified spectrophotometrically by measuring absorption at 260 nm and 280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and overall quality was assessed by gel electrophoresis. Samples were stored at -75ºC for future use.

Real-time quantitative RT-PCR (qRT-PCR). qRT-PCR was used to quantify various mRNAs using an LightCycler® 480 Real-Time PCR System (Hoffmann-La Roche Inc.) and the Brilliant II SYBR Green Single-Step QRT-PCR Master Mix (Agilent Technologies), with 10 ng of total RNA in 20 µl reaction mixtures. The thermal profile was 50°C for 30 min, 95°C for 15 min, and then 40 cycles of 95°C for 30 s and 55°C for 30 s. Fluorescence was measured at the end of the 55°C stage of each cycle. Samples were run in triplicate along with the no-reverse-transcriptase control and the no-template control. All experiments were repeated twice.

Fish infection experiments. Various *V. anguillarum* strains were tested for virulence against rainbow trout by immersion infection. Briefly, *V. anguillarum* cells grown for 19 h at 27°C in LB20 supplemented with the appropriate antibiotics were harvested by centrifugation (6,000 × *g*, 10 min, 4°C), washed twice in NSS, and resuspended in NSS. The *V. anguillarum* NSS suspension was prepared to the desired specific cell density according to the conversion equation: Cell density (10^8 CFU/ml) = 44.905 × OD_{600}. The

actual cell density of the suspension was confirmed by dilution and viable plate count. All fish were examined and determined to be disease and injury free prior to the start of each experiment. For immersion, 10 ml of *V. anguillarum* suspended in NSS, or 10 ml of NSS only as a negative control was added to a bucket filled with 10 L of water supplemented with 1.5% NaCl that was maintained at 18.5 ± 0.5ºC. Fish that were between 15 cm and 25 cm long were added and immersed for 1 h. For both methods, fish inoculated with different bacterial strains were maintained in separate 38 L or 191 L tanks to prevent possible cross-contamination with constant water flow (200 ml/min) at 19 ± 1ºC. Death due to vibriosis was determined by the observation of gross clinical symptoms and confirmed by the recovery and isolation of *V. anguillarum* cells resistant to the appropriate antibiotics from the spleen or head kidney of dead fish. Observations were made for 7 days. All fish used in this research project were obtained from the Lafayette Trout Hatchery located in North Kingstown, Rhode Island. All fish infection protocols were approved by the URI IACUC. (IACUC Protocol AN06-08-002).

Statistical analysis. Student's T-tests assuming unequal variances were used for statistical analyses for experiments containing two data groups. One-way ANOVA with repeated measures plus Tukey post hoc test was performed on bacterial growth curves. A Kaplan-Meier survival analysis with log rank significance test was performed on the survival percentage in the fish infection experiment. *P* values of <0.05 were considered statistically significant.

Results

Δ*gltA* mutant exhibited significant lower cell density limit than wild type in rich media and is auxotrophic for glutamate. Fig 1A shows a typical growth curve for the

wild type (M93Sm) and the Δ*gltA* mutant composed of a lag phase, exponential growth phase and stationary phase grown in a rich medium (dashed lines). Like the previously described *icd* mutant, the *gltA* mutant has a lower cell density limit rich media [28]. The *gltA* mutant is auxotrophic for glutamate because it cannot run the first half of the oxidative TCA cycle which is needed to synthesize the immediate precursor molecule for glutamate production, α-ketoglutarate (Fig 1B). When glutamate (118 mM final concentration) was added to LB20 (solid lines), the Δ*gltA* mutant grew to a wild type cell density (Fig 1A).

Δ*gltA* mutant exhibited either same or higher expression levels of the three hemolysin genes compared to wild type. As previously described, M93Sm secretes three hemolysins, Vah1, RtxA, and Plp that are most strongly expressed during exponential phase [11]. The expression of *vah1*, *rtxA* and *plp* was tested by qRT-PCR during exponential phase to determine if the Δ*gltA* mutant would have altered expression of these hemolysin genes. While there was no statistically significant increase in expression (*P* values ranged from 0.08 to 0.42), the *gltA* mutant exhibited 1.3-fold greater *rtxA* expression, 1.9-fold greater *plp* expression and 3.7-fold greater *vah1* expression compared to the wild type (Fig. 2).

Δ*gltA* mutant was as virulent as the wild type against juvenile rainbow trout. Ten juvenile rainbow trout were challenged via immersion at doses of 3×10^6 to 4×10^6 CFU/ml with the wild type (M93Sm) and the Δ*gltA* mutant. There was no significant difference in survival between M93Sm (10%) and the Δ*gltA* mutant (20%) ($P = 0.31$) (Table 3); however, it took three days for the Δ*gltA* mutant to reach 80% mortality compared to two days for 90% mortality in fish infected with M93Sm. While the data

strongly suggest that deleting *gltA* had a very small effect on virulence, the delay in mortalities raised the possibility that a mutation that compensated for the loss of citrate synthase was selected in the infected fish.

A mutation in *prpR* allowed the Δ*gltA* mutant to grow in 3M + 0.15% glucose. In order to test for the possibility that a compensatory mutation that would bypass the Δ*gltA* mutation could have been selected, the Δ*gltA* mutant was grown in 3M + 0.15% glucose for multiple days. An Δ*icd* mutant was also created and was unable to grow in 3M+0.15% glucose (data not shown). The Δ*gltA* mutant began to grow after 48 h, reaching wild type levels by 72 h (Fig. 3). A frozen stock was created from the 72 h culture. A single colony, isolated from the frozen stock, was used for another growth experiment in 3M + 0.15% glucose. This new strain was able grow in 3M + 0.15% glucose during a 24 h incubation. When the *gltA* gene was sequenced from this strain, there was no change in the sequence from the original Δ*gltA* mutation; it remained truncated.

It has previously been described that 2-methylcitrate synthase (PrpC), an enzyme that combines propionyl-COA and oxaloacetate to synthesize 2-methylcitrate, can act in a promiscuous manner and synthesize citrate from acetyl-CoA and oxaloacetate [34]. Accordingly, *prpB* (2-methylisocitrate lyase), *prpC, prpR* (transcriptional regulator) and the intergenic regions were sequenced using the primers listed in Table 2. A single nucleotide mutation was found in *prpR* that changed amino acid 66 from an arginine to a leucine. The wild type and the parental Δ*gltA* mutant did not have this mutation. No mutations were found in *prpB, prpC* or the intergenic regions. The spontaneous mutant was designated as Δ*gltA prpR* (R66L).

Δ*gltA prpR*(R66L) mutant has a higher expression level of *prpC* compared to wild type and the Δ*gltA* mutant. Digianantonio *et al* [35] demonstrated that increased expression of *prpC* enabled an *E. coli* Δ*gltA* mutant to grow on minimal media agar. Accordingly, the expression of *prpC* was measured by qRT-PCR during exponential phase to determine if the mutation found in the transcriptional regulator *prpR* of the Δ*gltA prpR*(R66L) mutant would cause an increase in expression of *prpC*. The Δ*gltA prpR*(R66L) mutant exhibited a 12- and 8-fold increase in expression compared to the wild type (M93Sm) and the Δ*gltA* mutant, respectively. These increases in *prpC* expression were statistically significant ($P<0.01$) demonstrating that the *prpR*(R66L) mutation caused an increase in expression of *prpC* (Fig. 4).

The Δ*gltA prpR*(R66L) mutant grew to a higher total cell density in spleen extract medium compared to the Δ*gltA* mutant. The final cell densities (CFU/ml) were determined for the wild type (M93Sm), the Δ*gltA* mutant and the Δ*gltA prpR*(R66L) mutant after 24 h of growth in three forms of rich media: LB20, NSSM and NSS supplemented with spleen extract (Table 4). In LB20 and NSSM, both the Δ*gltA* mutant and the Δ*gltA prpR*(R66L) mutants grew to a total cell density that was statistically lower than M93Sm. The difference between the Δ*gltA* mutant and the Δ*gltA prpR*(R66L) mutant was not statistically significant. In the spleen extract medium, M93Sm grew to the highest cell density (7.7×10^7 CFU/ml) followed by the Δ*gltA prpR*(R66L) mutant (1.5×10^7 CFU/ml), while the Δ*gltA* mutant grew to 5.7×10^7 CFU/ml. The difference between M93Sm and the Δ*gltA prpR*(R66L) mutant was not statistically significant. The data strongly suggest that the Δ*gltA prpR*(R66L) mutant had a growth advantage in spleen extract medium compared to the Δ*gltA* mutant.

The *prpR*(R66L) mutation in the Δ*gltA* mutant can be selected for in spleen extract medium. The wild type (M93Sm), the Δ*gltA* mutant and the Δ*gltA prpR*(R66L) mutant were grown in spleen extract medium for multiple days (Fig. 5). By 96 h, number of viable cells in the cultures of both M93Sm and the Δ*gltA prpR*(R66L) mutant began to decline. However, the cell density of the Δ*gltA* mutant increased between 96 h and 120 h. Colonies were chosen at random from the Δ*gltA* mutant 120 h agar plate and screened to ensure that the Δ*gltA* mutation was still deleted and if the colonies could grow in 3M + 0.15% glucose. All colonies had the truncated version of *gltA* while 4 out of the 6 colonies grew in 3M + 0.15% glucose with only a 24 h incubation. Although, it was not screened, the ability to grow 24 h in minimal media (Fig. 5) was presumably from a mutation in *prpR*. The data suggest that the *prpR*(R66L) mutation in the Δ*gltA* mutant strain is favored in spleen extract medium.

Δ*gltA* Δ*prpC* mutant is unable to grow in 3M + 0.15% glucose and grows to a lower cell density limit in two forms of rich media. A Δ*prpC* mutant and a Δ*gltA* Δ*prpC* mutant were created and their growth was examined. After 24 h, the Δ*gltA* Δ*prpC* mutant grew to a lower cell density in LB20 compared to the wild type (M93Sm) (Fig. 6A) and failed to grow in 3M + 0.15% glucose after 192 h (Fig. 6B). Growth in spleen extract medium was monitored and after 210 h the Δ*gltA* Δ*prpC* mutant failed to grow to the cell density of M93Sm or the Δ*gltA* mutant (Fig. 6C). The Δ*prpC* mutant grew to a wild type cell density in all forms of media (Fig. 6). The data demonstrate that when *prpC* is deleted in the Δ*gltA* mutant, the bacteria are no longer able to grow in 3M+0.15% glucose and lose their growth advantage in spleen extract media. However, the loss of *prpC* alone had little effect on growth.

Δ*gltA* Δ*prpC* double mutant is as virulent as the wild type against juvenile rainbow trout. Five to nine juvenile rainbow trout were challenged via immersion at a dose of 4×10^6 CFU/ml to 6×10^6 CFU/ml with either the wild type (M93Sm), the Δ*prpC* mutant, or the Δ*gltA* Δ*prpC* mutant. Fish infected with either M93Sm or the *prpC* mutant had 0% survival after 3 and 2 days, respectively (Table 5). While fish infected with the Δ*gltA* Δ*prpC* mutant had 22% survival, there was no significant difference in survival between M93Sm (0%) and the Δ*gltA* Δ*prpC* mutant (P = 0.23). The data suggest that deleting both *gltA* and *prpC* causes only a small attenuation of virulence.

Discussion

Citrate synthase catalyzes the first of eight reactions in the generation of energy through the oxidation of acetate by the tricarboxylic acid (TCA) cycle. TCA intermediates also serve as precursor metabolites for the synthesis of amino acids and peptidoglycan. Specifically, this investigation focused on the inability of the Δ*gltA* mutant to produce the TCA cycle intermediate and immediate precursor to glutamate synthesis, α-ketoglutarate. Previously, a *V. anguillarum icd* mutant was shown to be both highly attenuated in virulence and immunoprotective in juvenile rainbow trout [28]. The loss of pathogenicity was hypothesized to have resulted from the inability to synthesize glutamate; the cells would stop replicating and be prevented from reaching the threshold needed to cause systemic infection after they exhausted glutamate from their host. Aromatic compound auxotroph mutants have been demonstrated to be highly attenuated since the 1980s [12-16]. More recent studies have demonstrated that intracellular pathogens containing TCA cycle mutations are attenuated for virulence if the specific mutations prevent growth using the available nutrients in the nutrient-poor phagosome

[17-27]. Since *V. anguillarum* is not an intracellular pathogen and cannot survive in macrophages, it would not be subject to this type of nutrient limitation [36]. Additionally, the initial infection site *V. anguillarum* M93Sm, an O2α serotype, is presumably the intestines (external necrotic lesions have never been observed in fish exposed to this strain, unpublished observations) where it grows in the glutamate rich intestinal mucus [7, 8, 37]. Therefore, the previously described link between glutamate auxotrophy and attenuation in virulence in M93Sm warranted further investigation [28].

A Δ*gltA* strain was created and showed the same growth phenotype as the previously described *icd* mutant (Fig. 1) [28]. Unlike the *icd* mutant, the *gltA* mutant was as virulent as the wild type against juvenile rainbow trout. However, death from systemic infection was delayed by about 1 day (Table 3) suggesting that a compensatory mutation that could bypass the citrate synthase deletion was being selected for in the Δ*gltA* mutant. Indeed, a Δ*gltA* strain capable of growth in 3M + 0.15% glucose media (a medium in which it should fail to grow) was isolated (Fig. 3). When this Δ*gltA* strain was sequenced a spontaneous mutation was found in *prpR*, the transcriptional activator of 2-methylcitrate synthase (*prpC*), that changed arginine 66 to a leucine (this strain was named Δ*gltA prpR*(R66L)). The normal enzymatic function of PrpC is to synthesize 2-methylcitrate from propionyl-CoA and oxaloacetate; however, it has been demonstrated that PrpC can act promiscuously, substituting acetyl-CoA for propionyl-CoA to synthesize citrate [34]. The ability of the Δ*gltA prpR*(R66L)) mutant to grow in 3M + 0.15% glucose media resulted from an increase in expression of *prpC* (Fig. 4). The increase in expression of *prpC* enabled the cells to bypass the *gltA* deletion, an observation previously described in an *E. coli* Δ*gltA* strain by Digianantonio *et al* [35].

The hypothesized route of infection for *V. anguillarum* M93Sm is through the anus of the fish where it damages the lining of gastrointestinal tract to enter the circulatory system where it accumulates in the spleen [7, 8]. Accordingly, the *in vitro* growth of the Δ*gltA prpR*(R66L) mutant was characterized in NSSM and spleen extract medium to represent the environment of early and mid-stage infections respectively (LB20 served as a control). The growth advantage of the Δ*gltA prpR*(R66L) mutant in spleen extract medium coupled with the isolation of spontaneous *prpR* mutants in the Δ*gltA* mutant after extended growth in spleen extract medium suggest that during infection with the Δ*gltA* mutant, mutations in *prpR* are selected for in the fish spleen allowing the Δ*gltA* mutant to cause systemic infection. To test this idea, a Δ*gltA* Δ*prpC* mutant was created and was shown to be unable to grow in minimal media and had no growth advantage in spleen extract. Surprisingly there was no statistically significant difference in survival in fish challenged with the wild type and the Δ*gltA* Δ*prpC* mutant indicating that glutamate auxotrophy in and of itself may not necessarily result in attenuation.

The data presented here suggest that our previously proposed hypothesis that simple starvation for glutamate results in attenuation of virulence must be modified. While the small decrease in mortality during infection of the Δ*gltA* Δ*prpC* mutant suggests glutamate auxotrophy may contribute to attenuation, it may not be the single cause of attenuation in virulence of the *icd* mutant. The oxidative branch of the TCA cycle is at the intersection of three regulatory molecules: acetyl-CoA, citrate and α-ketoglutarate. Citrate accumulation has been shown to be detrimental to the growth rate and growth yield of *E. coli* with spontaneous citrate synthase mutants outgrowing *icd* or

aconitase (*acnA* and *acnB*) double mutants during *in vitro* growth experiments in complex media [38, 39]. Additionally, in *Staphylococcus aureus*, citrate has been shown allosterically activate CcpE, a regulator of metabolism and virulence factors [40]. An *E. coli icd* mutant was shown to have increased expression of the glyoxylate shunt causing lower acetyl-CoA production [41]. During acetogenesis, phosphotransacetylase converts acetyl-CoA into acetyl-P, a global regulator that is used to phosphorylate two-component signal transduction pathways [42]. Acetyl-P has been shown to induce virulence factor expression in *V. cholerae*, *S. enterica* and *E. coli* [43-46]. Further, acetyl-CoA accumulation alone, without being converting to acetyl-P, was shown to cause increased expression of the *V. cholerae* virulence gene activator ToxT [47]. Lastly, α-ketoglutarate has been shown to be a master regulator that coordinates nitrogen and carbon metabolism by inhibiting the production of cyclic AMP and has been shown to regulate virulence factors in UPEC [48-50]. Mutations in *gltA* or *icd* in *V. anguillarum* would result in altered intracellular levels of acetyl-CoA, citrate and α-ketoglutarate. The phenotype of the *icd* mutant (low intracellular levels of acetyl-CoA and high intracellular levels of citrate) could be causing the attenuation of virulence against juvenile rainbow trout because the cells can no longer sense their metabolic environment and consequently cannot properly coordinate the expression of appropriate virulence factors *in vivo*.

While expression levels of the three hemolysins in the *icd* and Δ*gltA* mutants have been examined and shown to be the same or higher compared to their expression wild type M93Sm cells *in vitro*, (Fig. 4) expression of other virulence factors important for systemic infection and persistence (the chemotaxic system, protease production, lipopolysaccharides production, iron acquisition, and flagella synthesis) have not been

examined and could be effected by the intracellular concentrations of acetyl-CoA, citrate, or α-ketoglutarate [28]. Further studies examining the entire transcriptome may reveal whether other potential virulence factors are affected by mutations in *icd* or *gltA* in *V. anguillarum*.

Conclusion

A *V. anguillarum* Δ*gltA* strain was created and was demonstrated to be similar to the previously described *icd* mutant; both strains demonstrated a decreased final cell density in multiple forms of rich media and had expression of hemolysin genes that was similar to the wild type. Unlike the *icd* mutant, the Δ*gltA* mutant was as virulent as the wild type in juvenile rainbow trout. Spontaneous mutations arose in *prpR* that caused an increase in expression of the promiscuous enzyme *prpC* allowing the Δ*gltA* mutant to bypass the *gltA* deletion and grow in minimal media and have a growth advantage in spleen extract media. A Δ*gltA* Δ*prpC* mutant was created and was demonstrated to be unable to grow in minimal media and no longer have a growth advantage in spleen extract media. However, the Δ*gltA* Δ*prpC* mutant exhibited only a slight attenuation in virulence compared to the wild type against rainbow trout further suggesting that simple starvation for a required nutrient (i.e. glutamate) will not directly result in attenuation of virulence as previously proposed.

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
V. anguillarum strains		
M93Sm	Spontaneous Sm^r mutant of M93 (serotype O2a)	[51]
ES496	Sm^r*; gltA* deletion mutant	This study
ES3-2	Sm^r*; gltA* deletion mutant, spontaneous mutation in *prpR* (R66L)	This study
ES6600	Sm^r*; prpC* deletion mutant	This study
ES496.6600	Sm^r*; gltA prpC* double mutant	This study
E. coli strains		
Sm10	*thi thr leu tonA lacY supE recA* RP4-2-Tc::Mu::Km (λ *pir*)	[52]
Q496	Km^r Cm^r; Sm10 containing plasmid pDM4-*gltA*5'-*gltA*3'	This study
Q6600	Km^r Cm^r; Sm10 containing plasmid pDM4-*prpC*5'-*prpC*3'	This study
Plasmid		
pDM4	Cm^r Km^r SacBC^r; suicide vector	[30]
pDM4-*gltA*5'-*gltA*3'	Cm^r SacBC^r; For *gltA* deletion mutant	This study
pDM4-*prpC*5'-*prpC*3	Cm^r SacBC^r; For *prpC* deletion mutant	This study

Table 2. Primers used in this study

Primer	Sequence (5'to3', lowercase sequences are designed to be homologous to flanking sequences)	Description[1]	Reference
PmG-01	caggttacccgcatgAGCTGCCGATTATGGATGGG	For *gltA* GA 5' region, forward	This study
PmG-02	ggaatgttaAAGTGAATCATGGTAGAACGCGG	For *gltA* GA 5' region, reverse	This study
PmG-03	gattcacttTAACATTCCTGAGTATGTGGTCAGAGC	For *gltA* GA 3' region, forward	This study
PmG-04	ctagatagatcttgcatgGGCCAGTATAAAGTTGACGAGGTC	For *gltA* GA 3' region, reverse	This study
PmG-23	aatcccgggagagctAACGCACCACTGGGCGGC	For *prpC* GA 5' region, forward	This study
PmG-24	tgaccaacGCCGGTGCGCATTACGTCC	For *prpC* GA 5' region, reverse	This study
PmG-25	gcaccggcGTTGGTCAAAAGCGTTATCTGAAGC	For *prpC* GA 3' region, forward	This study
PmG-26	cgggtaacctgagctTTAACGCTGCTCTATTGGTATCCAC	For *prpC* GA 3' region, reverse	This study
PmG-27	GCTCGATAGCATGCAAACCCGT	For *prpB* sequencing, forward	This study
PmG-28	CCGACAATCTGCAGCGGATCAT	For *prpB* sequencing, reverse	This study
PmG-29C	CTGCTCTATTGCCGTGCGAA	For *prpC* checking, forward	This study
PmG-30C	CGAGATCGCGCTTACGCTCA	For *prpC* checking, reverse	This study
Pm112s	AGCCCGATATCGCGCAGTTAAATG	For *gltA* checking, forward	This study
Pm117	TTCTTCTGGTGCTAACCCGTTTG	For *gltA* sequencing	This study
Pm118	GCTCTCGGAGAATGGTTAGCGA	For *gltA* checking, reverse	This study
vah1F RT	GTTTGGTATGGAACACCGCTCAAG	For *vah1* qRT-PCR, forward	[28]
vah1R RT	GGCTCAACCTCTCCTTGTAACCAA	For *vah1* qRT-PCR, reverse	[28]
plp F RT	CAGACGACCACCAGTAACCACTAA	For *plp* qRT-PCR, forward	[5]
plp R RT	GCAATCATGATGACCCAGCAACAG	For *plp* qRT-PCR, reverse	[5]
Pm111	GGAAATTATTCCGCCGACGATGGA	For *rtxA* qRT-PCR, forward	[4]
Pm112	GCCGATACCGTATCGTTACCTGAA	For *rtxA* qRT-PCR, reverse	[4]
Pm132	TTATGTACTGTCGGAAAAACTGGG	For *prpC* qRT-PCR, forward	This study
Pm133	TGGTCAAGTTCACTTTGATTAGGT	For *prpC* qRT-PCR, reverse	This study

[1]GA; Gibson Assembly

Table 3 Virulence of *V. anguillarum* strains in juvenile rainbow trout[1]

Strain	Dosage (CFU/ml)[2]	Total Mortality	No. of days until death (No. of fish / total)
M93Sm	4×10^6	90%	1 (3/10) 2 (9/10)
Δ*gltA*	$3\text{-}4\times10^6$	80%	1 (1/10) 2 (6/10) 3 (8/10)
Control (NSS)		0%	NA

[1] Sum of two treatments, five fish per treatment
[2] Δ*gltA* dosage: first treatment (3×10^6), second treatment (4×10^6)

Table 4 Final CFU/ml **(**±S. D) of various *V. anguillarum* cultures grown for 24 h[1]

Strain	CFU/ml in LB20	CFU/ml in NSSM (200 µg/ml)	CFU/ml in spleen extract (100 µg/ml)
M93Sm	3.4 (±0.3) $\times 10^9$ [1.00]	4.2 (±1.0) $\times 10^9$ [1.00]	7.7 (±2.1) $\times 10^7$ [1.00]
Δ*gltA*	1.6 (±0.1) $\times 10^9$ [0.47]**	1.2 (±0.2) $\times 10^9$ [0.29]*	1.5 (±0.05) $\times 10^7$ [0.19]*
Δ*gltA* *prpR*(R66L)	1.5 (±0.09) $\times 10^9$ [0.45]**	1.5 (±0.2) $\times 10^9$ [0.36]*	5.7 (±0.4) $\times 10^7$ (0.74)

S.D = standard deviation

[1]Values in brackets represent growth percentage compared to wild type.

** statistically significant to wild type ($P < 0.01$), * statistically significant to wild type ($P < 0.05$)

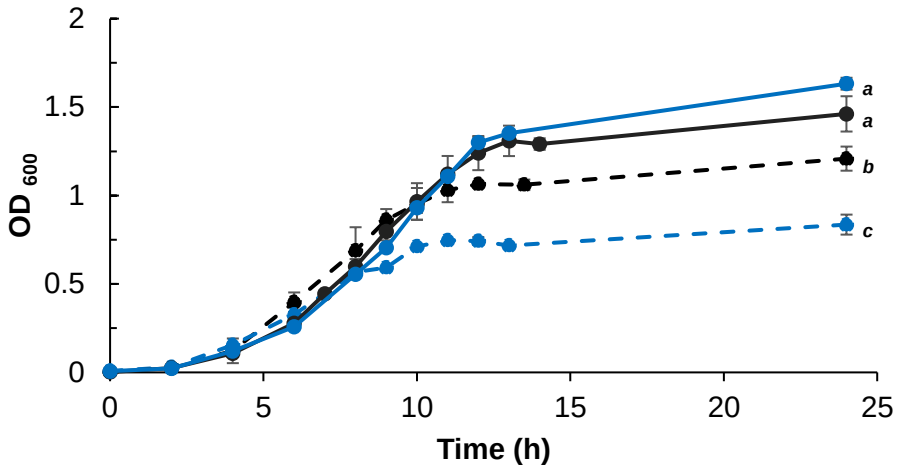
Table 5 Virulence of *V. anguillarum* strains in juvenile rainbow trout

Strain	Dosage (CFU/ml)	Total Mortality	No. of days until death (No. of fish / total)
M93Sm	5×10^6	100%	1 (6/9) 2 (8/9) 3 (9/9)
Δ*prpC*	6×10^6	100%	1 (2/5) 2 (5/5)
Δ*gltA* Δ*prpC*	4×10^6	78%	1 (4/9) 2 (6/9) 3 (7/9)
NSS		0%	3 (1/5)*

*Death was not caused by vibriosis

Figure 1. Growth of *V. anguillarum* WT (M93Sm) and the *gltA* mutant under various conditions. **A)** Growth curves of *V. anguillarum* M93Sm (black) and the *gltA* mutant (blue) in LB20 (dashed lines) or LB20 supplemented with 118 mM glutamate (solid lines). **B)** Final cell densities (OD_{600}) of *V. anguillarum* strains after 24 h of growth in 3M plus 0.15% glucose supplemented with or without 5.9 mM glutamate. In each experiment cells grown overnight in LB20 were washed twice in NSS, calibrated to an OD_{600} between 0.4 and 0.5 and used to inoculate the appropriate media at a 1:100 dilution. Cultures were incubated at 27^{o}C in a shaking water bath (200 rpm) and at various time points after inoculation samples were taken for determination of optical density at 600 nm (OD_{600}). The data are the average of two independent experiments. Different letters indicate statistical significance among groups ($P < 0.05$). Statistical analysis was based on data at 24 h. Between marked strains and M93Sm: ** $P < 0.01$. Error bars represent 1 standard deviation.

A


2
1.5
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Time (h)
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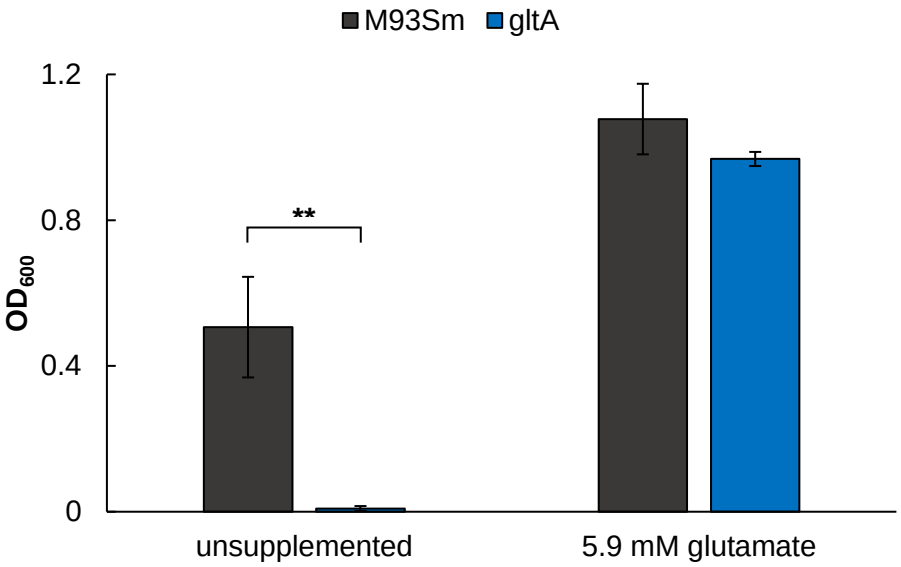

M93Sm
gltA
1.2
0.8
0.4
0
OD600
**
unsupplemented
5.9 mM glutamate

Figure 2. Expression of *vah1*, *rtxA*, and *plp* determined by qRT-PCR analysis of *V. anguillarum* wild-type (M93Sm) and the *gltA* mutant during logarithmic (Log)-phase growth. The data presented are the averages of two independent experiments. Each value is the average for six replicates. Error bars represent 1 standard deviation.

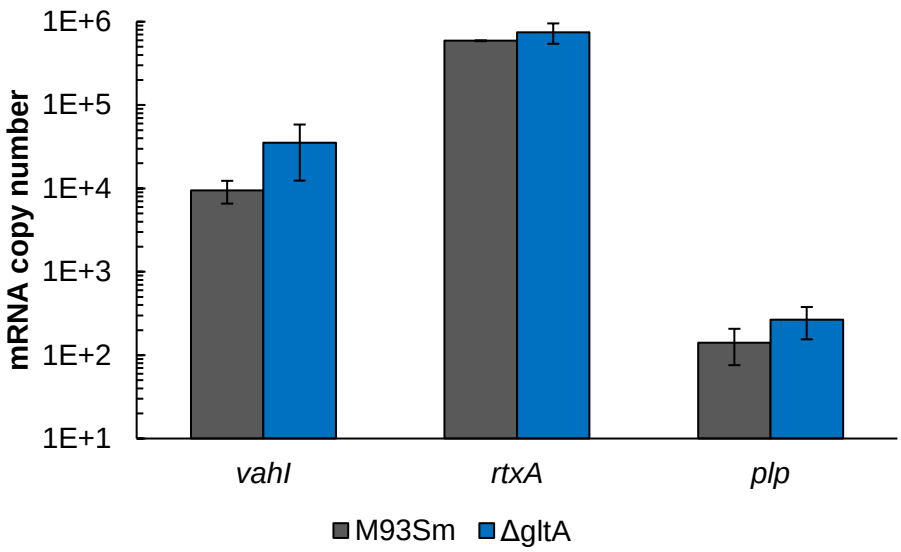

1E+6
1E+5
1E+4
1E+3
1E+2
1E+1
mRNA copy number
vahI
rtxA
plp
M93Sm
ΔgltA

Figure 3. Growth curves of various *V. anguillarum* strains grown in 3M + 0.15% glucose at 27°C with shaking (200 rpm). At various time points after inoculation samples were taken for determination of optical density at 600 nm (OD_{600}). In each experiment cells grown overnight in LB20 were washed twice in NSS, calibrated to an OD_{600} between 0.4 and 0.5 and used to inoculate the appropriate media at a 1:100 dilution. Cultures were incubated at 27^{o}C in a shaking water bath (200 rpm) and at various time points after inoculation samples were taken for determination of optical density at 600 nm (OD_{600}). The data are the average of two independent experiments. Error bars represent 1 standard deviation.

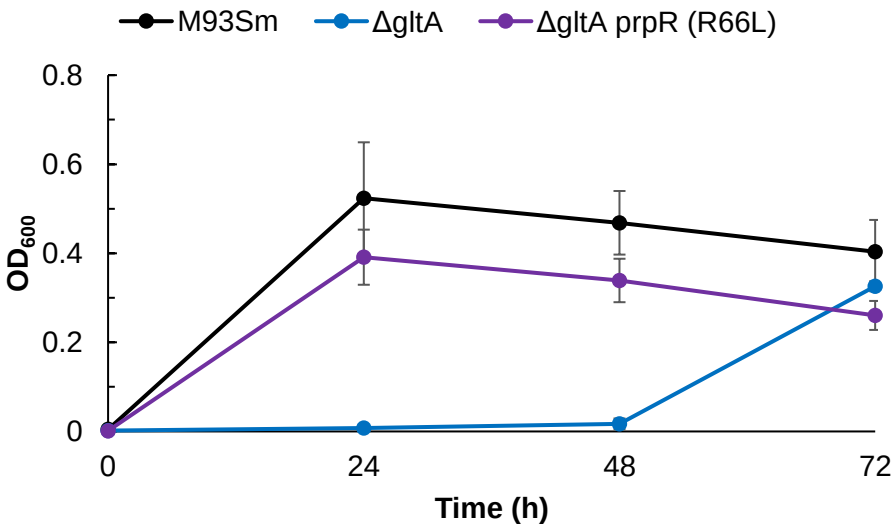

M93Sm
ΔgltA
ΔgltA prpR (R66L)
0.8
0.6
0.4
0.2
0
OD600
0
24
48
72
Time (h)

Figure 4. Expression of *prpC* determined by qRT-PCR analysis of *V. anguillarum* wild-type (M93Sm), the *gltA* mutant and the *gltA* mutant with the spontaneous mutation in *prpR*(R66L) during logarithmic (Log)-phase growth. The data presented are the averages of two independent experiments. Each value is the average of six replicates. Between marked strains and M93Sm: ** $P < 0.01$. Error bars represent 1 standard deviation.

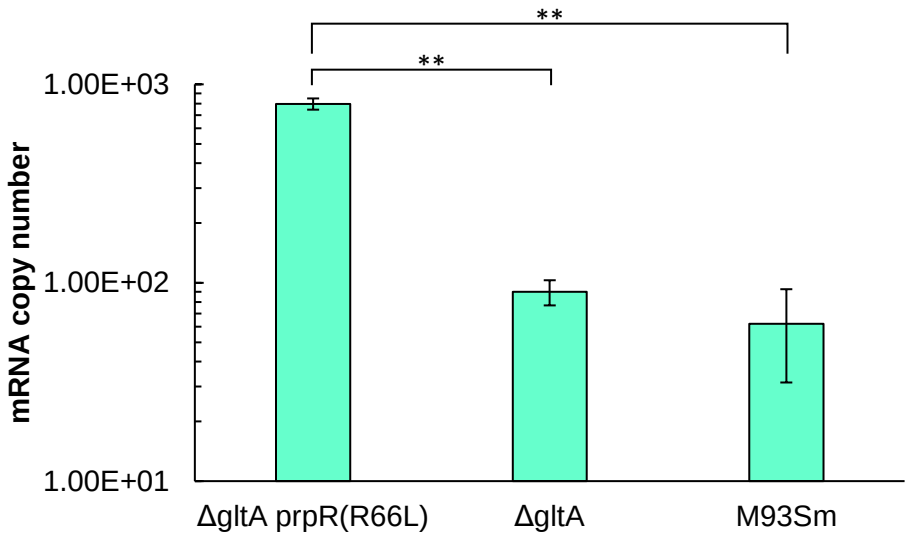

**
**
1.00E+03
1.00E+02
1.00E+01
mRNA copy number
ΔgltA prpR(R66L)
ΔgltA
M93Sm

Figure 5. Growth curves of various *V. anguillarum* strains grown in in NSS + spleen extract (100µg/ml) at 27°C with shaking (200 rpm). The data are a representative of two independent experiments. At various time points after inoculation samples were taken for determination CFU/ml by serial dilution and spot plating. In each experiment cells grown overnight in LB20 were washed twice in NSS, calibrated to an OD_{600} between 0.4 and 0.5 and used to inoculate the appropriate media at a 1:100 dilution. Δ*gltA* colonies were picked from the 120 h plate and used to inoculate 3M + 0.15% glucose. In addition, colony PCR using primers flanking *gltA* was performed to ensure *gltA* was still deleted.

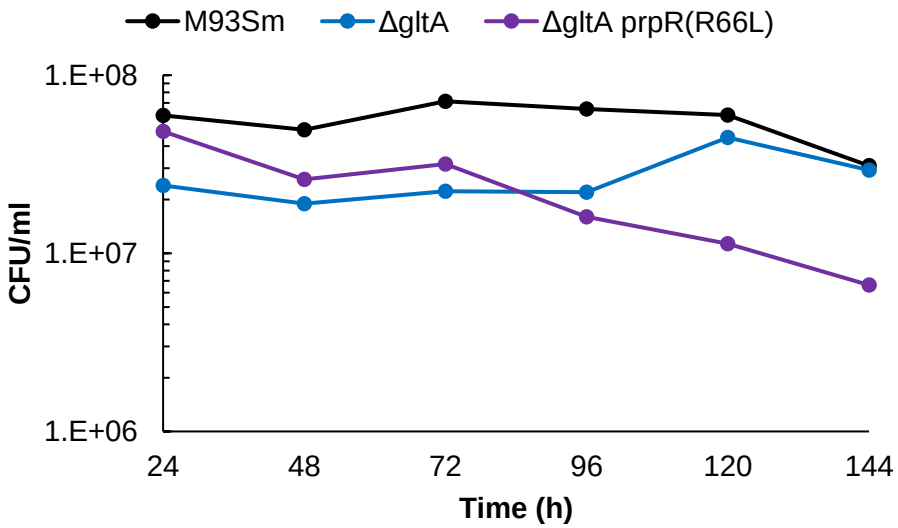
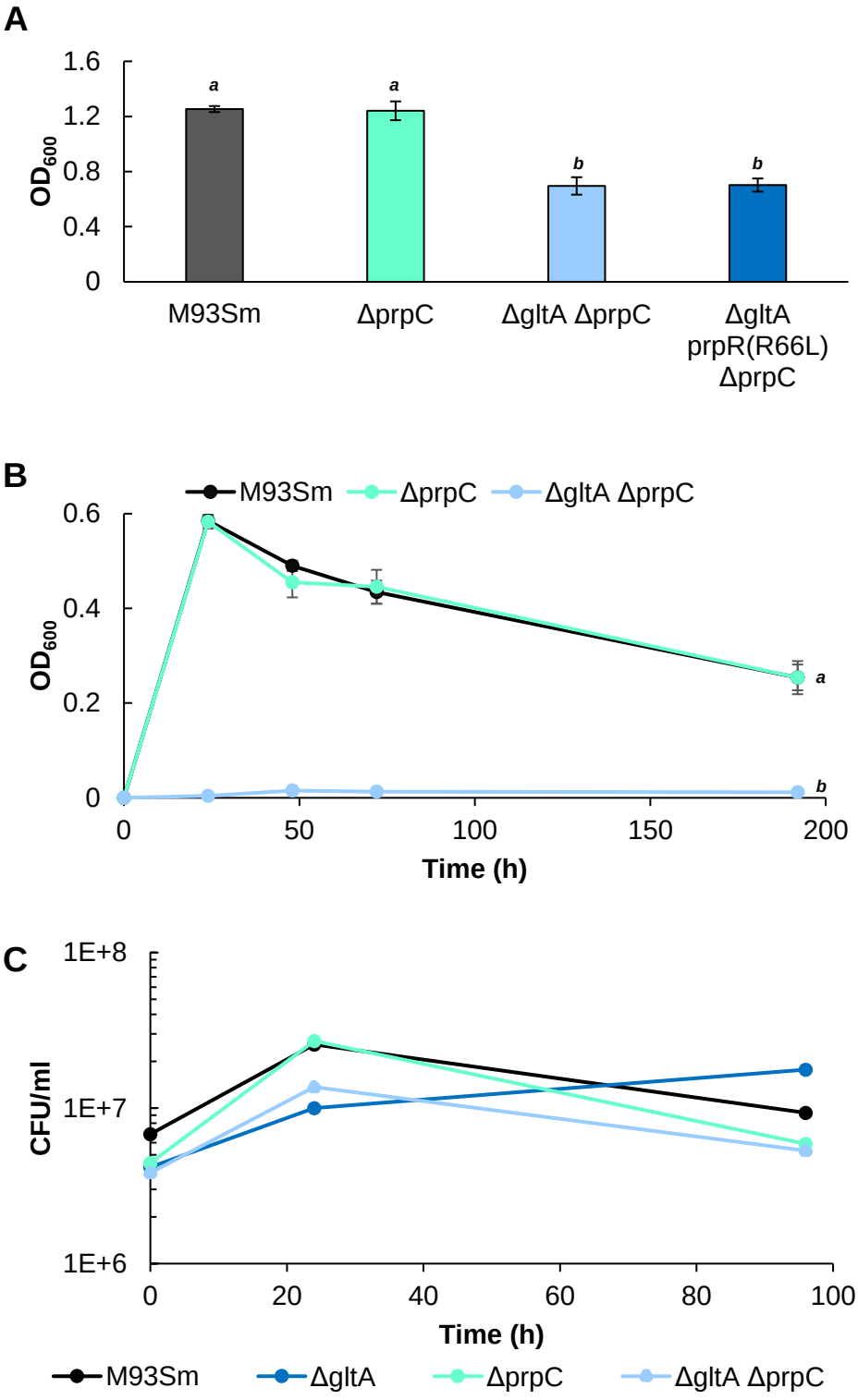

M93Sm
ΔgltA
ΔgltA prpR(R66L)
1.E+08
1.E+07
1.E+06
CFU/ml
24
48
72
96
120
144
Time (h)

Figure 6. Growth curves of various *V. anguillarum* strains grown in in various conditions at 27°C with shaking (200 rpm). **A)** Final cell densities (OD_{600}) of *V. anguillarum* strains after 24 h of growth in LB20. **B)** Growth curves of various *V. anguillarum* strains grown in 3M + 0.15% glucose at 27°C with shaking (200 rpm). **C)** Growth curves of various *V. anguillarum* strains grown in spleen extract medium at 27°C with shaking (200 rpm). Δ*gltA* colonies were picked from the 120 h plate, screened to ensure *gltA* was still deleted by colony PCR using primers flanking *gltA* and were used to inoculate 3M + 0.15% glucose. At various time points after inoculation samples were taken for determination of CFU/ml by serial dilution and spot plating or for determination of optical density at 600 nm (OD_{600}). In each experiment cells grown overnight in LB20 were washed twice in NSS, calibrated to an OD_{600} between 0.4 and 0.5 and used to inoculate the appropriate media at a 1:100 dilution. The data for A and B are the average of two independent experiments. Different letters indicate statistical significance among groups ($P < 0.05$). Statistical analysis was based on data at 24 h. Error bars represent 1 standard deviation.


A
1.6
1.2
0.8
0.4
0
OD600
a
a
b
b
M93Sm
ΔprpC
ΔgltA ΔprpC
ΔgltA prpR(R66L) ΔprpC
B
M93Sm
ΔprpC
ΔgltA ΔprpC
0.6
0.4
0.2
0
OD600
a
b
0
50
100
150
200
Time (h)
C
1E+8
1E+7
1E+6
CFU/ml
0
20
40
60
80
100
Time (h)
M93Sm
ΔgltA
ΔprpC
ΔgltA ΔprpC

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Manuscript-III

Prepare for submission to BMC Microbiology

Characterization of *Vibrio anguillarum* NB10Sm TCA cycle mutants

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Abstract

Background: There are twenty-three serotypes of the marine pathogen *Vibrio anguillarum*¸ three of which are responsible for causing warm water vibriosis, a fatal hemorrhagic septicemic disease of fish. A previous study demonstrated that a *V. anguillarum* M93Sm (O2a serotype) *icd* mutant was attenuated in virulence; however, it is not known if mutating this gene in the O1 serotype *V. anguillarum* NB10Sm will result in the same phenotype.

Results: A *V. anguillarum* NB10Sm *icd* mutant was created, characterized for growth in complex media and demonstrated to be as virulent as the wild type. Several additional central metabolism single and double mutants were created in the following genes *cra, gltA, Δicd gltA, sucA, sucC, sdhC, ΔfrdA, ΔfrdA sdhC,* and *fumA* and characterized with regard to growth in complex media. Two mutants (Δ*sucA* and Δ*frdA* Δ*sdhC*) that demonstrated a significantly reduced growth yield compared to the wild type were further characterized with regard to their growth is several forms of complex media, expression of virulence genes, and virulence in juvenile rainbow trout (*Oncorhynchus mykiss*).

Conclusions: The data strongly suggest that there is no correlation between a lower growth yield *in vitro* and a decrease in virulence *in vivo*. Even though M93Sm and NB10Sm are same species, mutations made in the same TCA cycle genes can cause drastically difference results in regards to growth and virulence.

Keywords: *Vibrio anguillarum*, TCA cycle, vibriosis, virulence, hemolysin, protease

Background

Vibrio anguillarum is a causative agent of warm water vibriosis, which causes a fatal hemorrhagic septicemic disease of fish, as well as morbidity and mortality in crustaceans and bivalves [1-3]. Mortality rates from vibriosis range between 30 to 100% and contribute to significant economic losses to the aquaculture industry worldwide [1, 3]. Typically, the initial site of infection is the skin, gills or intestines and *in vivo* studies have demonstrated that death from system infection usually occurs 1-4 days post infection [4-9]. Numerous virulence factors that have been shown to be important during infection include extracellular proteases, hemolytic cytotoxins, iron acquisition systems (siderophores), lipopolysaccharides, chemotaxis, and flagella [10].

Previously, a *V. anguillarum* M93Sm *icd* mutant was shown to be highly attenuated against juvenile rainbow trout and immunoprotective [11]. Auxotrophic or TCA cycle mutants attenuated in virulence have been created in several intracellular bacteria species [12-27]. The attenuation of the *V. anguillarum* M93Sm mutant was hypothesized to have resulted from the mutant being unable to grow to a final wild type cell density. The decreased growth yield resulted from the cells being auxotrophic and starved for glutamate [11]. If this same growth limitation were to occur during an infection, the cells would not be able to surpass threshold needed to cause disease. Interestingly, unlike the previously reported attenuated auxotrophic or TCA cycle mutants, *V. anguillarum* is not an intracellular pathogen and would not be experience the nutrient limitations of the phagosome [28]. Accordingly, glutamate auxotrophy may not be the single cause of the attenuation of the *icd* mutant as a later study demonstrated that

another mutant auxotrophic for glutamate (Δ*gltA*) was as virulent as the wild type [Spinard, EJ *et al*, in preparation, chapter 2 of this manuscript].

There are twenty-three serotypes of *V. anguillarum* (O1-O23) that have been differentiated by O-serotyping; only three serotypes (O1-O3) are important pathogens in fish [29, 30]. M93Sm is a spontaneous streptomycin resistant mutant selected from an O2a serotype strain originally isolated from a diseased ayu (*Plecoglossus altivelis*) from Lake Biwa in Japan [31]. Even though the M93Sm *icd* mutant stimulated an adaptive immune response that protected the fish from a subsequent M93Sm wild type challenge, it may be necessary to create a multivalent vaccine in order to promote full immunoprotection against multiple serotypes of *V. anguillarum*. In the late 1980s, salmon reared in New Brunswick vaccinated with bacterins composed of *V. anguillarum* serotype O1 and *V. ordalii* later died from vibriosis caused by *V. anguillarum* O2 serotypes; addition of an O2 bacterin reduced death from *V. anguillarum* O2 serotype induced vibriosis [32]. Accordingly, this study focused on creating and characterizing the growth and virulence of central metabolism mutants in *V. anguillarum* NB10Sm (spontaneous streptomycin resistant O1 serotype strain originally isolated from a rainbow trout near the city of Boden, Sweden) with the goal of creating a live attenuated O1 strain [33].

Methods

Bacterial strains, plasmids, and growth conditions. *V. anguillarum* strains (Table 1) were routinely grown in Lysogeny broth containing 2% NaCl (LB20) [34], LB20 containing various additional supplements, Marine Minimum Median (3M) + 0.15% glucose [35], NSS supplemented with fish gastrointestinal mucus (NSSM), NSS supplemented with fish skin mucus (NSSSkM) or NSS Spleen extract medium

supplemented with the appropriate antibiotic, in a shaking water bath at 27°C. *E. coli* strains (Table 1) were routinely grown in Lysogeny broth containing 1% NaCl (LB10) supplemented with the appropriate antibiotic, in a shaking water bath at 37°C. Antibiotics were used at the following concentrations: streptomycin, 200 μg/ml (Sm^{200}); chloramphenicol, 20 μg/ml (Cm^{20}) for *E. coli* and 5 μg/ml (Cm^{5}) for *V. anguillarum*; 40μg/ml (Km^{40}) kanamycin for liquid media or 80μg/ml (Km^{80}) kanamycin for solid media for *V. anguillarum* and *E. coli*.

Insertional mutagenesis. Insertional mutations in the target genes: *icd*, *sucA*, *sucC*, *sdhC*, *fumA* and *cra* were created by using previously constructed suicide vectors [11]. Additional insertional mutations were made by using a modification of the procedure described by Milton *et al.* [36]. Briefly, primers (Table 2) were designed based on the target gene sequence of NB10. A 200-300 bp DNA fragment of the target gene was PCR amplified and ligated into the suicide vector pNQ705-1 (Table 1) after digestion with SacI and XbaI. The ligation mixture was introduced into *E. coli* SM10 by electroporation using a BioRad Gene Pulser II (BioRad, Hercules, CA). Transformants were selected on LB10 Cm^{20} agar plates. The construction of the recombinant pNQ705 was confirmed by PCR amplification. The mobilizable suicide vector was transferred from *E. coli* SM10 into *V. anguillarum* by conjugation [37]. Transconjugants were selected by utilizing the chloramphenicol resistance gene located on the suicide plasmid. The incorporation of the recombinant pNQ705 was confirmed by PCR amplification.

Allelic exchange mutagenesis. Deletion mutations were made by using a modification of the procedure described by Milton *et al.* [36]. Suicide vectors were constructed via restriction enzyme digestion/ligation (*frdA*, *sucA*, *icd*, CUO) or Gibson Assembly (*sdhC*).

Briefly, primers (Table 2) were designed based on the target gene sequence of NB10 (Accession numbers: NZ_LK021130.1, NZ_LK021129.1 and NZ_LK021128.1). For restriction enzyme digestion and ligation, a DNA fragment of the 5' region of the target gene varying in size from 297 to 425 bp was PCR amplified and ligated into the suicide vector pDM4 (Table 1) after digestion with SacI and XbaI. Subsequently a section of the 3' region of the target gene varying in size from 273 to 484 bp was PCR amplified and ligated into the suicide vector pDM4 containing the previously added 5' region after digestion with XhoI and NheI. Finally, the kanamycin resistance cassette was amplified and ligated into the previously constructed suicide vector after digestions with XbaI and XhoI. For the Gibson Assembly method [38], primers (Table 2) were designed based on the target gene sequence of NB10. A 100 bp DNA fragment of the 5' region and the 3' region of the target gene was PCR amplified and ligated using the Gibson assembly method into the suicide vector pDM4 (Table 1) previously digested with SacI. The ligation or the Gibson Assembly mixture was introduced into *E. coli* SM10 by electroporation using a BioRad Gene Pulser II (BioRad, Hercules, CA). Transformants were selected on LB10 Cm^{20} agar plates. The construction of the recombinant pDM4 was confirmed by PCR amplification. The mobilizable suicide vector was transferred from *E. coli* SM10 into *V. anguillarum* by conjugation. Single-crossover transconjugants were selected with LB20 Sm^{200} Cm^{5} Km^{80} plates, and subsequently, double-crossover transconjugants were selected with LB20 Sm^{200} Km^{80} plates containing 5% sucrose. The resulting *V. anguillarum* mutants were checked for the desired allelic exchange by PCR amplification using primers (Table 2) flanking the deletion.

Cell growth experiments. *V. anguillarum* cells grown overnight at 27°C in LB20 supplemented with the appropriate antibiotics were harvested by centrifugation (9,000 × *g*, 2 min), washed twice and resuspended in in NSS. A 200 µl aliquot of the *V. anguillarum* NSS suspension was transferred into a 96-well plate with a clear flat bottom and the optical density at 600 nm (OD_{600}) was read by a VersaMax™ Absorbance Microplate Reader (Molecular Devices). The *V. anguillarum* NSS suspension was prepared to an OD_{600} between 0.300 and 0.350 (~1.5×10^9 CFU/ml) and diluted 1:100 into fresh media. Growth was monitored either by measurement of the OD_{600} or by serial dilution and plate counts.

Fish infection experiments. Various *V. anguillarum* strains were tested for virulence against rainbow trout by immersion infection. Briefly, *V. anguillarum* cells grown for 20 h at 27°C in LB20 supplemented with the appropriate antibiotics were harvested by centrifugation (6,000 × *g*, 10 min, 4°C), washed twice in NSS, and resuspended in NSS. The *V. anguillarum* NSS suspension was prepared to the desired specific cell density according to the conversion equation derived from Supplementary Figure 1: Cell density = $(5 \times 10^9 \times OD_{600}) - 4 \times 10^7$. The actual cell density of the suspension was confirmed by dilution and viable plate count. All fish were examined and determined to be disease and injury free prior to the start of each experiment. For immersion, 10 ml of *V. anguillarum* suspended in NSS, or 10 ml of NSS only as a negative control was added to a bucket filled with 10 L of water supplemented with 1.5% NaCl that was maintained at 19.5 ± 0.5ºC. Fish that were between 15 cm and 25 cm long were added and immersed for 1 h. For both methods, fish inoculated with different bacterial strains were maintained in separate 191 L tanks to prevent possible cross-contamination with constant water flow

(200 ml/min) at 19 ± 1ºC. Death due to vibriosis was determined by the observation of gross clinical symptoms and confirmed by the recovery and isolation of *V. anguillarum* cells resistant to the appropriate antibiotics from the spleen or head kidney of dead fish. Observations were made for 7 days. All fish used in this research project were obtained from the Lafayette Trout Hatchery located in North Kingstown, Rhode Island. All fish infection protocols were approved by the URI Institutional Animal Care and Use Committee (IACUC) (IACUC Protocol AN06-08-002).

RNA isolation. Exponential phase cells (~3×10^8 CFU/ml) or stationary phase cells (1.0×10^9 to 7.8×10^9 CFU/ml) of various *V. anguillarum* strains were treated with RNAprotect Bacteria Reagent (QIAGEN), following the manufacturer's instructions. Total RNA was isolated using the RNeasy kit following the instructions of the manufacturer. All purified RNA samples were quantified spectrophotometrically by measuring absorption at 260 nm and 280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and overall quality was assessed by gel electrophoresis. Samples were stored at -75ºC for future use.

Real-time quantitative RT-PCR (qRT-PCR). qRT-PCR was used to quantify various mRNAs using an LightCycler® 480 Real-Time PCR System (Hoffmann-La Roche Inc.) and the Brilliant II SYBR Green Single-Step QRT-PCR Master Mix (Agilent Technologies), with 10 ng of total RNA in 20 µl reaction mixtures [7]. The thermal profile was 50°C for 30 min, 95°C for 15 min, and then 40 cycles of 95°C for 30 s and 55°C for 30 s. Fluorescence was measured at the end of the 55°C stage of each cycle. Samples were run in triplicate along with the no-reverse-transcriptase control and the no-template control. All experiments were repeated twice.

Statistical analysis. Student's T-tests assuming unequal variances were used for statistical analyses for all experiments except for the fish infection experiment. *P* values of <0.05 were considered statistically significant). A Kaplan-Meier survival analysis with log rank significance test was performed on the survival percentage in the fish infection experiment. *P* values of <0.05 were considered statistically significant.

Results

***icd* mutant grew to the same cell density limit as wild type in complex media.** A *V. anguillarum* M93Sm *icd* mutant was previously shown to grow to a lower cell density than the wild type in nutrient rich media (e.g. LB20 and NSSM) [11]. A *V. anguillarum* NB10Sm *icd* insertional mutant was constructed and Fig. 2A shows a typical growth curve for the wild type (NB10Sm) and the *icd* mutant. Unlike the previously described M93Sm *icd* mutant, the NB10Sm *icd* had a slower growth rate during mid and late exponential phase growth compared to the wild type but reached the same cell density after growth for 24 h (Table 3, Supplementary Fig. 4) [11]. No additional copies of *icd* are found in the genome and the NB10Sm *icd* mutant was unable to grow in minimal media indicating that there is no promiscuous enzyme that can act as an isocitrate dehydrogenase (Fig. 2B). To ensure the *icd* insertional mutant (a merodiploid) did not resolve to restore the wild type, an *icd* deletion strain was created and showed no difference in growth compared to the *icd* insertional mutant (Fig. 2C). The data suggest that unlike the M93Sm *icd* mutant, the NB10Sm *icd* mutant can obtain enough α-ketoglutarate derivatives (e.g. glutamate, glutamine, and peptides containing those amino acids) from LB20 to grow to a wild type final cell density.

***icd* mutant exhibits at a wild type growth rate when LB20 is supplemented with gluconate or glutamate.** Fig. 3 shows the typical growth curve of the wild type (NB10Sm) (black) and the *icd* mutant (blue) in LB20 not supplemented (Fig. 3, dashed lines) or supplemented with 101.7 mM gluconate (Fig. 3A, solid lines) or 118 mM glutamate (Fig. 3B, solid lines). The data indicate that the *icd* mutant exhibited a growth rate during the mid and late exponential growth phases similar to the wild type when LB20 was supplemented with a metabolite that feeds into central metabolism upstream (gluconate) or downstream (glutamate) of the *icd* mutation (Table 3). Interestingly, the wild type grew to a higher cell density than the *icd* mutant when LB20 was supplemented with gluconate. The data suggests that under these conditions the *icd* mutant will utilize gluconate to grow rapidly but will experience a cell density limitation when the cells exhaust the available glutamate.

A ΔCUO *icd* mutant has the same growth phenotype as the *icd* mutant. The CUO (citrate utilization operons) is a ~17.9 kbp region of the NB10 genome, consisting of multiple genes and operons: a citrate lyase operon (accession numbers: WP_013856981.1, WP_013856980.1, WP_013856979.1), an ACP synthase (accession number: WP_013856979.1), an oxaloacetate decarboxylase operon (accession numbers: WP_013856986.1 and WP_029189993.1), a chemotaxis protein and kinase (accession number: WP_013856983.1 and WP_013856984.1), and a tri-carboxylate importer operon (accession numbers: WP_013856993.1, WP_019281353.1, and WP_013856991.1). The CUO is not present in the M93Sm genome and may provide an alternative to the glyoxylate shunt to prevent the intracellular buildup of citrate. Interestingly, the upstream genes (accession numbers: WP_013856977.1, WP_017049831.1, WP_019282875.1, and

WP_019282876.1) and downstream genes (accession numbers: WP_017046166.1 and ASO29090.1) flanking this region in the NB10Sm genome are homologous to neighboring genes in the M93Sm genome indicating an occurrence of a gain of function (NB10Sm) or loss of function (M93Sm) (Supplementary Fig. 2). The CUO region was deleted to determine if it enabled the NB10Sm *icd* mutant grow to a final cell density that was similar to the wild type. Fig. 4 shows a typical growth curve of the wild type (NB10Sm), the *icd* mutant, the ΔCUO mutant and the ΔCUO *icd* mutant. The ΔCUO mutant had a growth curve nearly identical to the wild type and the ΔCUO *icd* mutant had a growth curve that was nearly identical to the *icd* mutant indicating that the presence of the CUOs did not enable the NB10Sm *icd* mutant to reach to a wild type cell density after 24 h.

***icd* mutant is as virulent as the wild type against juvenile rainbow trout.** To determine if the NB10Sm *icd* mutant would be as virulent as the wild type (NB10Sm), 10 juvenile rainbow trout were challenged via immersion at a dose of between 6.5×10^6 CFU/ml to 9.4×10^6 CFU/ml (Table 4). Although it took seven days for the *icd* mutant to cause 70% morality and five days for the wild type to cause 80% mortality, there was no statistically significant difference in virulence between the two strains (P= 0.30).

***sucA* mutant, *fumA* mutant, and a Δ*frdA sdhC* mutant grow to a final cell density in complex media lower than the wild type.** It was previously proposed that a decreased final cell density in complex media resulting from a nutrient limitation *in vitro* could correlate to attenuation in virulence *in vivo* [11]. Since the NB10Sm *icd* mutant did not show either a decrease in final cell density or a loss of virulence, an additional ten central metabolism single and double mutants were created in the following genes: *cra, gltA,*

Δicd gltA, sucA, sucC, sdhC, ΔfrdA, ΔfrdA sdhC, and *fumA* (Table 1). Fig. 5A shows a typical growth curve of the wild type (NB10Sm) and various central metabolism mutants. Examination of the growth yield for the various mutant strains after 24 h by optical density shows that the *sucA* mutant (OD_{600} = 0.551), *fumA* mutant (OD_{600} = 0.618), and a Δ*frdA sdhC* mutant (OD_{600} = 0.482) failed to grow to a wild type (OD_{600} = 1.08) cell density (Fig. 5B). The *sucA* mutant and the Δ*frdA sdhC* mutant have a large standard deviation at the 24 h time point that could have resulted from the strains resolving the merodiploid present in the *sucA* or *sdhC* gene. Supplementary Fig. 3 demonstrates how this reversion to a wild type phenotype could occur when the insertional mutant strains are grown in the absence of the antibiotic chloramphenicol.

Supplementing LB20 with additional nutrients restores growth of the Δ*frdA* Δ*sdhC* mutant but not the Δ*sucA* mutant to wild type levels. Fig 6A shows the final cell density (OD_{600}) of the wild type (NB10Sm), Δ*frdA* Δ*sdhC* mutant and the Δ*sucA* mutant after growth for 24 h and 48 h in LB20. The deletion mutations were shown to be stable because the standard deviation between biological repeats was nearly nonexistent and there was no increase in growth between 24 h and 48 h. The two mutant strains were further screened via colony PCR using primers specific to the pJM1 plasmid to ensure the decreased growth yield did not result from loss of this plasmid (data not shown). Fig 6B shows the final cell density (OD_{600}) of the wild type and the Δ*frdA* Δ*sdhC* mutant grown in LB20 or LB20 supplemented with succinic acid (118 mM), gluconate (118 mM), malate (118 mM) or aspartate (118 mM). The data indicate that the addition of malate or aspartate increased the growth of the Δ*frdA* Δ*sdhC* mutant to near wild type level. Although the Δ*frdA* Δ*sdhC* mutant cannot convert succinate to fumarate, it is not

auxotrophic for any metabolite. Fig. 6C shows the 24 h cell density (OD_{600}) of the wild type and Δ*sucA* mutant grown in LB20 or LB20 supplemented with lysine (118 mM) or DL-α, ε-diaminopimelic acid (118 mM) or lysine, DL-α, ε-diaminopimelic acid, and methionine (118 mM). The Δ*sucA* mutant is auxotrophic for the TCA cycle intermediate succinyl-CoA, a metabolite that is needed in diaminopimelic acid, lysine and methionine biosynthesis [40]. The addition of lysine, DL-α, ε-diaminopimelic acid and methionine to LB20 did not restore the growth of the Δ*sucA* mutant to a wild type cell density and indicates that the cells are either unable to import lysine or DL-α, ε-diaminopimelic acid or methionine or the cells require metabolites upstream of diaminopimelic acid in the biosynthetic pathway of lysine and the enzymes involved in this pathway cannot run in reverse. Interestingly, the addition of lysine and DAP actually caused the wild type to grow to a lower cell density, indicating that these cells do not favor these metabolites for growth.

The Δ*sucA* mutant and the Δ*frdA* Δ*sdhC* mutant grow to a lower cell density compared to the wild type in various types of complex media. The final cell densities (CFU/ml) of the wild type (NB10Sm), Δ*sucA* mutant and the Δ*frdA* Δ*sdhC* mutant were determined after 24 h of growth in four forms of complex media: LB20, NSSM, Spleen extract medium, and NSSSkM (Table 6). In all forms of complex media, the Δ*sucA* mutant grew to the lowest cell density followed by the Δ*frdA* Δ*sdhC* mutant.

All mutants exhibited either same or lower expression levels of the three hemolysin genes compared to wild type. Vah1, RtxA, and Plp are three hemolysins secreted by *V. anguillarum* M93Sm that have been characterized and determined to be responsible for the hemolytic/cytolytic activity against fish erythrocytes, leukocyte and epithelial cells [7,

9, 41] and unpublished data]. It is unknown how these hemolysins contribute to the virulence of *V. anguillarum* NB10Sm. The expression of *vah1*, *rtxA* and *plp* during exponential phase was tested to determine whether mutations in metabolism could affect the expression of these hemolysin genes (Fig. 7A). *rtxA* was the most highly expressed (>4.6×10^3 copies/10 ng RNA) of the three hemolysins in NB10Sm and there was no statistically significant difference between the wild type (NB10Sm) and the two mutants (Δ*sucA* and Δ*frdA* Δ*sdhC*). The mutants have a decrease in expression in both *plp* and *vah1* compared to the wild type. However, these genes were expressed at the limit of detection by qRT-PCR. In the wild type, there were <30 copies/10 ng RNA for *plp* and <10 copies/10 ng RNA for *vah1*.

All mutants exhibited lower levels of *empA* metalloprotease gene expression compared to wild type. It has previously been demonstrated that *V. anguillarum* NB10 *empA* metalloprotease mutants are slightly attenuated in virulence and that *empA* expression is dependent upon RpoS and, therefore, highly expressed during stationary phase [6, 42, 43]. The expression of *empA* during stationary phase was tested to determine whether mutations in metabolism could affect the expression of this gene (Fig. 7B). There was a ~3000-fold decrease in the expression of *empA* in the Δ*sucA* mutant and a 23-fold decease in the Δ*frdA* Δ*sdhC* mutant compared to the wild type (NB10Sm). Both decreases in expression were statistically significant ($P = 0.008$ and $P = 0.01$ respectively). The data indicate that mutations in central metabolism can cause a decrease in the expression of the metalloprotease *empA.*

Δ*sucA* mutant and the Δ*frdA* Δ*sdhC* mutant are as virulent as the wild type against juvenile rainbow trout. To determine if the Δ*sucA* mutant and the Δ*frdA* Δ*sdhC* mutant

would be as virulent as the wild type (NB10Sm), 5 juvenile rainbow trout were challenged via immersion at a dose of between 1.0×10^6 CFU/ml to 4.0×10^6 CFU/ml (Table 7). There was no statistically significant difference in mortality between fish challenged with the wild type (100%) and the Δ*sucA* mutant (100%) (P= 0.39) or Δ*frdA* Δ*sdhC* mutant (100%) (P= 0.65). The data once again indicate that central metabolism mutants that have a lowered final cell density due to a starvation of a nutrient will not necessarily be attenuated in virulence.

Discussion

A *V. anguillarum* M93Sm *icd* mutant (O2α serotype) was previously shown to be attenuated in virulence and elicit an immunoprotective response in juvenile rainbow trout [11]. Though, *V. anguillarum* is an extracellular pathogen, the attenuation was thought to have resulted from the inability of the mutant to synthesize α-ketoglutarate and its amino acid derivatives (i.e. glutamate and glutamine). Subsequently, we demonstrated that a M93Sm Δ*gltA* mutant, also auxotrophic for glutamate, exhibited no reduction in virulence *in vivo*. Unlike the previously reported intracellular pathogens, the attenuation in virulence of the *icd* mutant is likely to involve more than just a simple nutrient starvation [12-27] [Spinard, EJ et al, in preparation, chapter 2 of this manuscript 2017]. Regardless of the cause of the attenuation of the M93Sm *icd* mutant, it was still immunoprotective and has potential as a live cell vaccine against vibriosis. *V. anguillarum* vaccines must be multivalent in order to fully protect against vibriosis caused by all *V. anguillarum* serotypes (O1, O2, O3) implicated in disease [30]. Accordingly, this study originally sought to create a live attenuated O1 serotype strain by mutating *icd* in *V. anguillarum* NB10Sm. The NB10Sm *icd* mutant (the insertional

mutant and the deletion mutant) did not show the cell density limitation previously observed in the M93Sm *icd* mutant in complex media (Fig. 2) nor was it reduced in virulence against juvenile rainbow trout (Table 4) [11]. The NB10Sm *icd* mutant (ES920 and ES926) did have a longer generation time than the wild type during mid and late exponential growth that was shortened by the addition of gluconate or glutamate to LB20. The data suggest that the decrease in growth rate of the NB10Sm *icd* mutant (ES920) occurs because the cells are required to utilize unfavorable metabolites or perform energetically unfavorable reactions during the mid and late exponential growth phase that can be alleviated by the addition of an extra energy source (gluconate) or metabolite that cannot be synthesized (glutamate). It is not clear why the *V. anguillarum* NB10Sm *icd* mutant is not starved for α-ketoglutarate like the M93Sm *icd* mutant. One explanation could be linked to the higher expression levels of the *empA* metalloprotease in *V. anguillarum* NB10 compared to *V. anguillarum* M93Sm in noninduced (LB20) conditions [6]. LB20 is composed of yeast extract and tryptone (both of which are composed of peptides of varying sizes), although bacteria can only utilize peptides that are six amino acids in size or smaller [44, 45]. The increase in expression of *empA* could allow the NB10Sm *icd* mutant (ES920) to more efficiently break down and utilize proteins and large sized peptides than M93Sm, preventing the cells from being starved for glutamate.

Since the NB10Sm *icd* mutant (ES920) was not attenuated in virulence and did not have a final cell density limit that was lower than the wild type after 24 h of growth in complex media, additional central metabolism mutants and double mutants were constructed (Table 1). Mutations in *fumA, sucA* or Δ*frdA sdhC* caused reductions in the

final cell density (Fig. 5). Focus was placed on the *sucA* mutant and the Δ*frdA sdhC* mutant and deletion mutants were constructed for each strain to prevent the mutants from spontaneously resolving the merodiploid (a phenomenon that was previously exploited to create the *icd* revertant strain [11]). The Δ*frdA ΔsdhC* mutant can run the reductive and oxidative branch of the TCA cycle to synthesize any intermediate and accordingly, is not auxotrophic for any metabolite when it is grown with glucose as the only carbon source. However, the data suggest that the cells favor metabolites that feed into the TCA cycle at α-ketoglutarate such as glutamate and glutamine. Since the TCA cycle can only run α-ketoglutarate to succinate (the reaction catalyzed by *icd* is nonreversible), the cells are starved for the amino acid aspartate. The growth limitation of the Δ*sucA* mutant was not fully characterized but is presumed to have resulted from the inability of the cells to synthesize succinyl-CoA, a metabolite utilized in the biosynthetic pathways for the production of diaminopimelic acid, lysine and methionine [40]. The initial hypothesized route of infection for *V. anguillarum* NB10Sm is through the skin, gills or anus of the fish where it enters the circulatory system and accumulates in the spleen [4, 5]. Accordingly, the final cell densities of the Δ*sucA* mutant and Δ*frdA ΔsdhC* mutant were determined in media that represent early infection (NSSM and NSSSkM) and mid-infection (spleen extract medium). In all forms of complex media (including LB20 as a control) the Δ*sucA* mutant grew to the lowest cell density, followed by the Δ*frdA ΔsdhC* mutant (Table 6). The data suggests that, even under ideal conditions at each point of the infection process, the mutants could be starved for essential metabolites *in vivo*. However, the infection experiments (Table 7) demonstrate that the starvation does not correlate to a loss of virulence *in vivo* (discussed below).

The expression of the virulence factors (*rtxA, plp, vah1, empA*) were examined in the Δ*sucA* mutant and Δ*frdA ΔsdhC* mutant and compared to the wild type (Fig. 7). NB10 has been previously been shown to be non-hemolytic on blood agar, presumably from the hemolysins being degraded by EmpA before they can lyse the red blood cells [9]. This study expands upon this explanation and shows that the actual expression levels of the hemolysins is much lower in NB10Sm compared to M93Sm. Expression of *rtxA* during log phase was relatively similar between the wild type and the mutants, although a decrease in expression was observed for *plp* and *vah1* (Fig. 7). This decrease was not believed to be relevant since the expression of *plp* and *vah1* was very low in the wild type (28 and 6.5 copies per 10 ng of total RNA, respectively). It has previously been shown that a *V. anguillarum* M93Sm *rtxA* mutant is avirulent [7]. It is not known how *rtxA* contributes to the virulence of *V. anguillarum* NB10Sm especially since the RtxA of M93Sm and NB10Sm have different effector domains [46]. This study demonstrates that the expression of *rtxA* is 127-fold lower than in M93Sm [Spinard, EJ et al, in preparation, chapter 2 of this manuscript]. Yet, the expression of NB10Sm *rtxA* was still 160-fold higher than the expression of NB10Sm *plp* suggesting that it could be important for the infection process of NB10Sm. Expression of the metalloprotease *empA* was examined during stationary phase. *empA* has been shown to be nonessential for the virulence of *V. anguillarum* NB10 [42, 47]. It has previously been demonstrated that the expression of *empA* is cell density dependent [6]. Accordingly, the Δ*sucA* mutant had the lowest final cell density (13% of the wild type) and the lowest expression level of *empA* (0.03% of the wild type). The transcript of VanT, the transcriptional activator of *empA,* is stabilized by the sigma factor RpoS [43, 47]. Alternatively, the decrease in expression of *empA* in the

mutants could have resulted from a decrease in expression of RpoS.

Regardless of the lowered cell density limit and the decrease in *empA* production, both the Δ*sucA* mutant and Δ*frdA ΔsdhC* mutant were as virulent as the wild type in juvenile rainbow trout (Table 7). The metabolite starvation that prevents the mutants from reaching a wild type growth yield *in vitro* does not prevent the cells from reaching the threshold needed to cause disease *in vivo*. Accordingly, the approach that was used in this study to find attenuated strains, screening central metabolism mutants that grow to a lower cell density than the wild type *in vitro*, was unsuccessful probably because *V. anguillarum* is an extracellular pathogen that grows in the nutrient rich mucus of the skin or intestines. Still, nutrient starvation must affect the infection process of *V. anguillarum* and contribute to attenuation of the previously reported M93Sm *icd* mutant (decreased final cell density compared to the wild type). If it did not, it would have been expected that the NB10Sm *icd* mutant (same final cell density as the wild type) would have been attenuated. Yet, there must be other unknown factors besides nutrient starvation that are contributing to the reduction of virulence seen in *V. anguillarum* M93Sm *icd* mutant or else the previously reported M93Sm Δ*gltA* mutant, that experiences the same starvation for glutamate as the *icd* mutant, would have been attenuated. Further studies must be performed with the M93Sm *icd* mutant to fully understand the cause of attenuation.

Conclusions

Multiple central metabolism mutants and double mutants were constructed in *V. anguillarum* NB10Sm. Unlike the previously described *V. anguillarum* M93Sm *icd* mutant, the NB10Sm *icd* mutant was as virulent as the wild type. Although both the Δ*sucA* mutant and Δ*frdA ΔsdhC* mutant grew to lower cell densities than the wild type in

several forms of complex media and had large and significant decreases in expression of the *empA* metalloprotease, neither strain was attenuated in virulence. Compared to *plp* and *vah1*, there was higher expression in the gene encoding the MARTX toxin *rtxA*, suggesting that *rtxA* may contribute to the virulence of NB10Sm. The data demonstrate that strain specific phenotypes may result even if mutations are made in the same highly conserved gene.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
V. anguillarum strains		
NB10Sm	Spontaneous Sm^r mutant of NB10 (serotype O1)	[42]
SM920	Sm^r Cm^r; *icd* insertional mutant	This study
ES926	Sm^r Km^r; *icd* allelic exchange mutant	This study
ES926.990	Sm^r Cm^r Km^r; *icd* allelic exchange, *gltA* insertional double mutant	This study
ES930	Sm^r Cm^r; *cra* insertional mutant	This study
ES940	Sm^r Cm^r; *sucA* insertional mutant	This study
ES946	Sm^r Km^r; *sucA* allelic exchange mutant	This study
ES950	Sm^r Cm^r; *sucC* insertional mutant	This study
ES960	Sm^r Cm^r; *sdhC* insertional mutant	This study
ES970	Sm^r Cm^r; *fumA* insertional mutant	This study
ES985	Smr Cm^r Km^r; *frdA* insertional mutant	This study
ES986	Sm^r Km^r; *frdA* allelic exchange mutant	This study
ES986.966	Sm^r Km^r; *frdA* allelic exchange, *sdhC* allelic exchange double mutant	This study
ES990	Sm^r Cm^r; *gltA* insertional mutant	This study
ES2546	Sm^r Km^r; CUO allelic exchange mutant	This study
ES2546.920	Sm^r Cm^r Km^r; CUO allelic exchange *icd* insertional double mutant	This study
E. coli strains		
Sm10	*thi thr leu tonA lacY supE recA* RP4-2-Tc::Mu::Km (λ *pir*)	[48]
S100	Km^r; Sm10 containing plasmid pNQ705-1	[49]
Q925	Km^r Cm^r; Sm10 containing plasmid pDM4-*icd*	This study
Q420	Km^r Cm^r; Sm10 containing plasmid pNQ705-*icd*	[11]
Q440	Km^r Cm^r; Sm10 containing plasmid pNQ705-*sucA*	[11]
Q450	Km^r Cm^r; Sm10 containing plasmid pNQ705-*sucC*	[11]
Q460	Km^r Cm^r; Sm10 containing plasmid pNQ705-*sdhC*	[11]
Q470	Km^r Cm^r; Sm10 containing plasmid pNQ705-*fumA*	[11]
Q430	Km^r Cm^r; Sm10 containing plasmid pNQ705-*cra*	[11]
Q990	Km^r Cm^r; Sm10 containing plasmid pNQ705-*gltA*	This study
Q945	Km^r Cm^r; Sm10 containing plasmid pDM4-*sucA*	This study
Q2545	Km^r Cm^r; Sm10 containing plasmid pDM4-CUO	This study
Q985	Km^r Cm^r; Sm10 containing plasmid pDM4-*frdA*	This study
Q965	Km^r Cm^r; Sm10 containing plasmid pDM4-*sdhC*	This study
Plasmid		
pNQ705-1	Cm^r; suicide vector with R6K origin	[49]
pDM4	Cm^r Km^r SacBC; suicide vector	[35]
pNQ705-*gltA*	Cm^r; For *gltA* insertional mutant	This study
pNQ705-*icd*	Cm^r; For *icd* insertional mutant	[14]
pNQ705-*sucA*	Cm^r; For *sucA* insertional mutant	[14]
pNQ705-*sucC*	Cm^r; For *sucC* insertional mutant	[14]
pNQ705-*sdhC*	Cm^r; For *sdhC* insertional mutant	[14]
pNQ705-*fumA*	Cm^r; For *fumA* insertional mutant	[14]
pNQ705-*cra*	Cm^r; For *cra* insertional mutant	[14]
pDM4-*icd*	Cm^r Km^r SacBC; For *icd* deletion mutant	This study
pDM4-*sucA*	Cm^r Km^r SacBC; For *sucA* deletion mutant	This study
pDM4-CUO	Cm^r Km^r SacBC; For CUO deletion mutant	This study
pDM4-*sdhC*	Cm^r SacBC; For *sdhC* deletion mutant	This study

Table 2. Primers used in this study.

Primer	Sequence (5' to 3')[1, 2]	Description	Reference
pr31	GGTGAGCTCTATTCTTTATTGCCGATTATC	For *icd* insertional mutant, forward, SacI	[14]
pr32	AAATCTAGAGTAAGTCGCTTTAATCGCTTC	For *icd* insertional mutant, reverse, XbaI	[14]
pr50	AAAGAGCTCGTGATCCAGATGTCGATGCTA	For *sucA* insertional mutant, forward, SacI	[14]
pr51	GGTTCTAGAGTTCAGTGTCGATAATGTGCA	For *sucA* insertional mutant, reverse, XbaI	[14]
pr52	AAAGAGCTCGGTCGGATTAGTACAGCGAAG	For *sucC* insertional mutant, forward, SacI	[14]
pr53	GGTTCTAGACTTTTTCAATTTCCACGCCGC	For *sucC* insertional mutant, reverse, XbaI	[14]
pr54	AAAGAGCTCATGTTCGTTGCGGTCGGAATT	For *sdhC* insertional mutant, forward, SacI	[14]
pr55	GGTTCTAGATCCAACTCTTCAAAGTGGCCC	For *sdhC* insertional mutant, reverse, XbaI	[14]
pr56	GGTGAGCTCTCCTTGCACCATATTGATATG	For *fumA* insertional mutant, forward, SacI	[14]
pr57	GGGTCTAGAAGGCTTATCATCGAGAAGAGAG	For *fumA* insertional mutant, reverse, XbaI	[14]
pm110	GGCGAGCTCTCGATGGCGAAAAAG	For *gltA* insertional mutant, forward, SacI	This study
pm111	GGCTCTAGAGCTCGTGGACCAT	For *gltA* insertional mutant, reverse, XbaI	This study
pr33	AAAGAGCTCGCGGCGTGAGACTAAGGCATC	For *cra* insertional mutant, forward, SacI	[14]
pr34	AAATCTAGACAATGGCAAAGCGCAGAAGTA	For cra insertional mutant, reverse, XbaI	[14]
pm119	GCCGAGCTCTTTGTTCTGCTGGCTTTTTA	For CUO 5' region, forward, sac1	This study
pm120	GCCTCTAGAGGGATTTTGTTGGAAGTTGA	For CUO 5' region, reverse xba1	This study
pm121	GGCCTCGAGCTTAACTGTCATTCAGCCTC	For CUO 3' region, forward, xhoI	This study
pm122	GGCGCTAGCGTGGAGGCTCTCTTTCTTAT	For CUO 3' region, reverse nheI	This study
PmG-13	*aatcccggga*GAGCTCCGTGAAAGAAAGAAAGTCAAGACCTG	For *sdhC* 5' region, forward, Gibson assembly	This study
PmG-14	*aaatggcg*ACCCCAGACACTCGGTGTAG	For *sdhC* 5' region, reverse, Gibson assembly	This study
PmG-15	*tctggggt*CGCCATTTGCTGATGGACC	For *sdhC* 3' region, forward, Gibson assembly	This study
PmG-16	*cgggtaacct*GAGCTCTAAAACTGAAAGCACTGCTGTTGC	For *sdhC* 3' region, reverse, Gibson assembly	This study
pr68	TTAATTTCTAGATGATAATGGTGCGCGGCGATC	For *icd* 5'region, reverse, xba1	This study
pr69	TAATAACTCGAGAAAGCACGGCGCATTTGATCC	For *icd* 3' region, forward, xho1	This study
pr70NheI	ACTGCTGCTAGCGAATAGATCCGTGAGGTAATC	For *icd* 3' region, reverse, nhe1	This study
pm64suc	GCACTCGAGTCTGGTTTGTTATCGCCGTC	For *sucA* 3' region, forward, xho1	This study
pm65suc	GTAGCTAGCGCGGATTCGCTTTCCATCAT	For *sucA* 3' region, reverse, nhe1	This study
pm76	GTAGAGCTCGGAGGCTCAGCAGCAGTTAT	For *frdA* 5' region, forward, sacI	This study
pm77	GATTCTAGAGAAGAGGGTGTGCAGCATGT	For *frdA* 5' region, reverse, xbaI	This study
pm78	CTACTCGAGTTCGCACTGAGATGGGTCAC	For *frdA* 3' region, forward, xhoI	This study
pm79	GTTGCTAGCTCGATACTTGGAGCGGCATC	For *frdA* 3' region, reverse, nheI	This study
pm38	CGGCTATCTAGAGAAAAGCTTGAACACGTAGAA	For KanR forward, XbaI	
pm39	GCATTACTCGAGCGTTTCTGGGTGAGCAAAAAC	For KanR reverse, xhoI	
vah1 F RT	GTTTGGTATGGAACACCGCTCAAG	For *vah1* qRT-PCR, forward	Reference
vah1 R RT	GGCTCAACCTCTCCTTGTAACCAA	For *vah1* qRT-PCR, reverse	Reference
plp F RT	CAGACGACCACCAGTAACCACTAA	For *plp* qRT-PCR, forward	Reference
plp R RT	GCAATCATGATGACCCAGCAACAG	For *plp* qRT-PCR, reverse	Reference
Pm111	GGAAATTATTCCGCCGACGATGGA	For *rtxA* qRT-PCR, forward	Reference
Pm112	GCCGATACCGTATCGTTACCTGAA	For *rtxA* qRT-PCR, reverse	Reference
empA-qRT-PCR-F	TCAAATCTTCCGGTGGCTTACGTT	For *empA* qRT-PCR, forward	Reference
empA-qRT-PCR-R	GCGCGATTAAACACACCACTTGAA	For *empA* qRT-PCR, reverse	Reference

[1]lowercase sequences are designed to be homologous to flanking sequences
[2]underlined sequences are designed to be restriction enzyme cut sites

Table 3. Generation times of various *V. anguillarum* strains grown in LB20[1]

Strain	Early Exponential Phase (Minutes)			Mid Exponential Phase (Minutes)			Late Exponential Phase (Minutes)		
	LB20	**Gluconate**	**Glutamate**	**LB20**	**Gluconate**	**Glutamate**	**LB20**	**Gluconate**	**Glutamate**
NB10Sm	55	65	74	98	84	84	105	99	102
icd (ES920)	55	63	68	108	76	83	107	97	101

[1]Values calculated from data presented in Figure 2A and Figure 3 during exponential growth.

Table 4. Virulence of *V. anguillarum* strains in juvenile rainbow trout

Strain	Dosage (CFU/ml)	Total Mortality	No. of days until death (No. of fish / total)
NB10Sm	7.2-9.4×10^6	80%	2 (6/10) 3 (5/10) 4 (6/10) 5 (8/10)
icd (ES920)	6.5-6.9×10^6	70%	2 (1/10) 3 (3/10) 4 (4/10) 5 (5/10) 7 (7/10)
NSS		0%	NA

Table 5. Generation times of various *V. anguillarum* strains grown in LB20[1]

Strain	Early Exponential Phase (Minutes)	Late Exponential Phase (Minutes)
NB10Sm	55	102
icd (ES920)	45	102
gltA	51	100
Δ*icd gltA*	54	103
cra	52	102
sucA	55	115
sucC	67	107
sdhC	58	109
fumA	57	108
Δ*frdA*	60	101
Δ*frdA sdhC*	58	113

[1]Values calculated from data presented in Figure 5A during exponential growth.

Table 6. Final cell density (CFU/ml) of various *V. anguillarum* cultures grown for 24 h

Strain	CFU/ml in LB20	CFU/ml in NSSM (200µg/ml)	CFU/ml in Spleen	CFU/ml in NSSSkM (200µg/ml)
NB10Sm	7.8×10^{9} ($\pm1.1\times10^{9}$)	7.2×10^{9} ($\pm0.9\times10^{8}$)	1.5×10^{8} ($\pm0.2\times10^{8}$)	1.6×10^{9} ($\pm0.3\times10^{8}$)
Δ*sucA*	1.0×10^{9} ($\pm0.9\times10^{9}$)**	4.6×10^{8} ($\pm2.7\times10^{8}$)**	1.2×10^{7} ($\pm0.8\times10^{7}$)**	3.8×10^{7} ($\pm0.3\times10^{8}$)**
Δ*frdA* Δ*sdhC*	2.2×10^{9} ($\pm0.6\times10^{9}$)*	2.5×10^{9} ($\pm1.2\times10^{9}$)*	7.7×10^{7} ($\pm0.5\times10^{7}$)	2.2×10^{8} ($\pm0.3\times10^{8}$)**

** statistically significant to wild type ($P < 0.01$), * statistically significant to wild type ($P < 0.05$)

Table 7. Virulence of *V. anguillarum* strains in juvenile rainbow trout

Strain	Dosage (CFU/ml)	Total Mortality	No. of days until death (No. of fish / total)
NB10Sm	4×10^6	100%	2 (3/5) 3 (4/5) 4 (5/5)
Δ*sucA*	3.5×10^6	100%	2 (1/5) 3 (4/5) 5 (5/5)
Δ*frdA* Δ*sdhC*	$1.\times10^6$	100%	2 (4/5) 3 (5/5)
NSS	NA	0%	NA

TABLE 8. Comparison of all mutant *V. anguillarum* strains

Strain Information				Growth Characteristics							Gene Expression					Virulent in rainbow trout
					Final Growth Yield											
Name	Gene	Mutation Type	Parental Strain	Growth Rate	LB20	3M + 0.15% Glucose	NSSM	Spleen	NSSSkM	Auxotrophic	*rtxA*	*plp*	*vah1*	*prpC*	*empA*	
XM410	*mdh*	ID	M93Sm	-	+	+	ND	ND	ND	ND	+	+	+	ND	ND	+
XM420	*icd*	ID	M93Sm	+	-	-	-	ND	ND	α-ketoglutarate derivatives	+	+	+	ND	ND	-
XM430	*cra*	ID	M93Sm	+	+	+	ND	ND	ND	ND	+	++	+	ND	ND	+
XM440	*sucA*	ID	M93Sm	-	+	-	ND	ND	ND	Succinyl CoA derivatives	+	++	++	ND	ND	+
XM450	*sucC*	ID	M93Sm	-	+	+	ND	ND	ND	ND	+	++	++	ND	ND	+
XM460	*sdhC*	ID	M93Sm	-	+	+	ND	ND	ND	ND	+	++	++	ND	ND	+
XM470	*fumA*	ID	M93Sm	-	+	+	ND	ND	ND	ND	+	+	++	ND	ND	+
ES422	*icd*	NA	XM420	+	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	+
ES496	*gltA*	AE	M93Sm	+	-	±	-	-	ND	α-ketoglutarate derivatives	+	+	+	+	ND	+
ES3-2	*gltA, prpR*	AE, R66L	M93Sm	+	-	+	-	+	ND	α-ketoglutarate derivatives	ND	ND	ND	++	ND	+
ES496.6600	*gltA, prpC, prpR*	AE, AE, R66L	M93Sm	+	-	-	-	-	ND	α-ketoglutarate derivatives	ND	ND	ND	ND	ND	+
ES6600	*prpC*	AE	M93Sm	+	+	+	ND	+	ND	ND	ND	ND	ND	ND	ND	+
ES920	*icd*	ID	NB10Sm	-	+	-	ND	ND	ND	α-ketoglutarate derivatives	ND	ND	ND	ND	ND	+
ES926	*icd*	AE	NB10Sm	-	+	ND	ND	ND	ND	α-ketoglutarate derivatives	ND	ND	ND	ND	ND	ND
ES926.990	*icd, gltA*	AE, ID	NB10Sm	+	+	ND	ND	ND	ND	α-ketoglutarate derivatives	ND	ND	ND	ND	ND	ND
ES930	*cra*	ID	NB10Sm	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ES940	*sucA*	ID	NB10Sm	-	-	ND	ND	ND	ND	succinyl CoA derivatives	ND	ND	ND	ND	ND	ND
ES946	*sucA*	ID	NB10Sm	ND	-	ND	-	-	-	succinyl CoA derivatives	+	-	-	ND	-	+
ES950	*sucC*	ID	NB10Sm	-	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ES960	*sdhC*	ID	NB10Sm	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ES970	*fumA*	ID	NB10Sm	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ES986	*frdA*	AE	NB10Sm	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ES986.960	*frdA, sdhC*	AE, ID	NB10Sm	-	-	ND	ND	ND	ND	aspartate	ND	ND	ND	ND	ND	ND
ES986.966	*frdA, sdhC*	AE, AE	NB10Sm	ND	-	ND	-	+	-	aspartate	+	+	-		-	+
ES990	*gltA*	ID	NB10Sm	+	+	ND	ND	ND	ND	α-ketoglutarate derivatives	ND	ND	ND	ND	ND	ND
ES2546	CUOs	AE	NB10Sm	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ES2546.920	CUOs *icd*	AE, ID	NB10Sm	-	+	ND	ND	ND	ND	α-ketoglutarate derivatives	ND	ND	ND	ND	ND	ND

ID: Insertional Deletion (merodiploid capable of restoring to a wild type genotype)
AE: Allelic Exchange
NA: Not Applicable
ND: Not Determined
CUO: Citrate Utilization Operon
+ Wild type level
++ Greater than wild type
- Less than Wild type

Figure Legends

Figure 1. Embden-Meyerhoff-Parnas Pathway, TCA cycle, and metabolism of fructose. The arrows indicate the physiological directions of the reactions. The gene symbols of the enzyme for each reaction are listed beside the reaction. Boxed genes indicate the genes that were mutated in this study (Table 1).

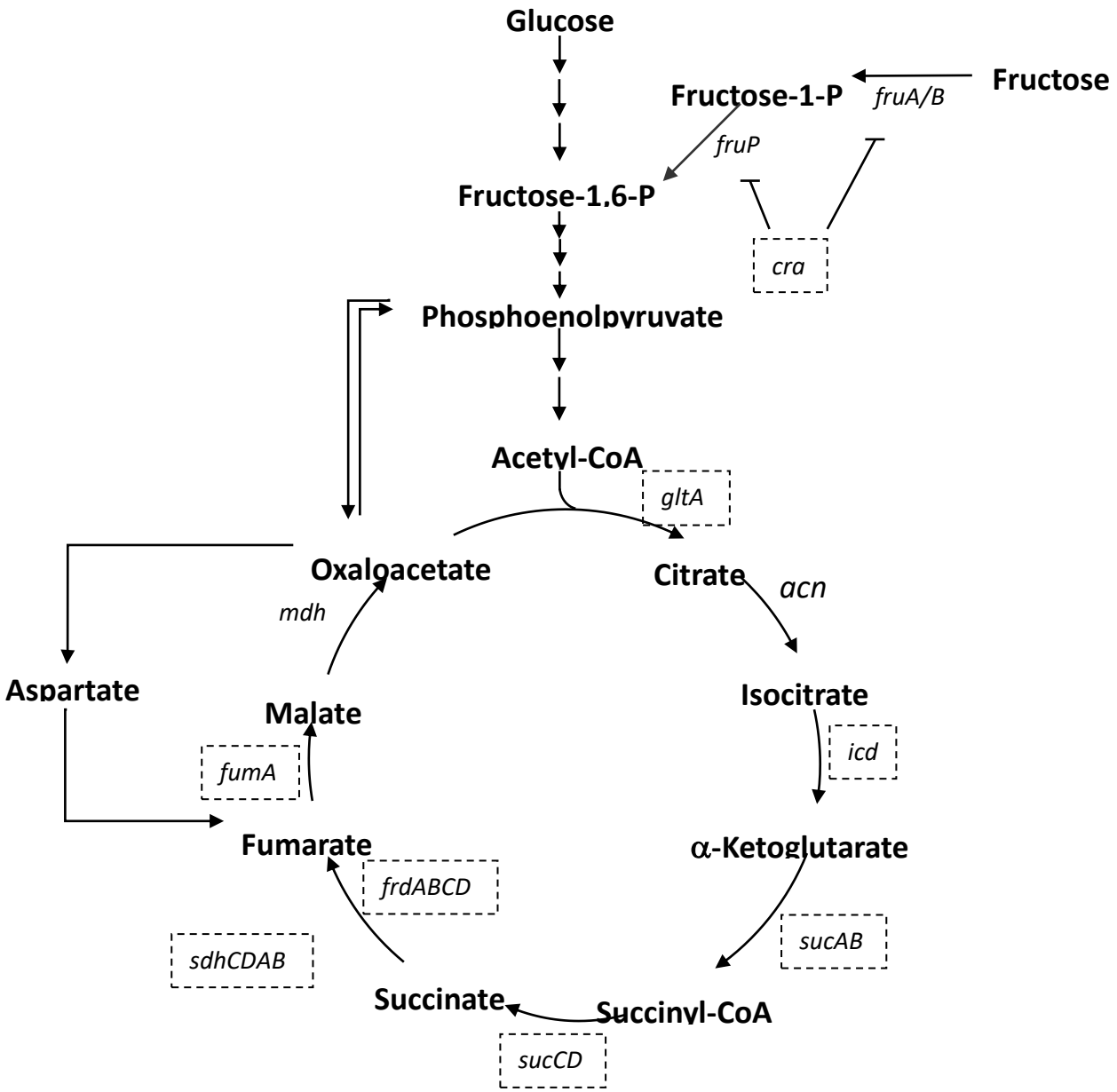
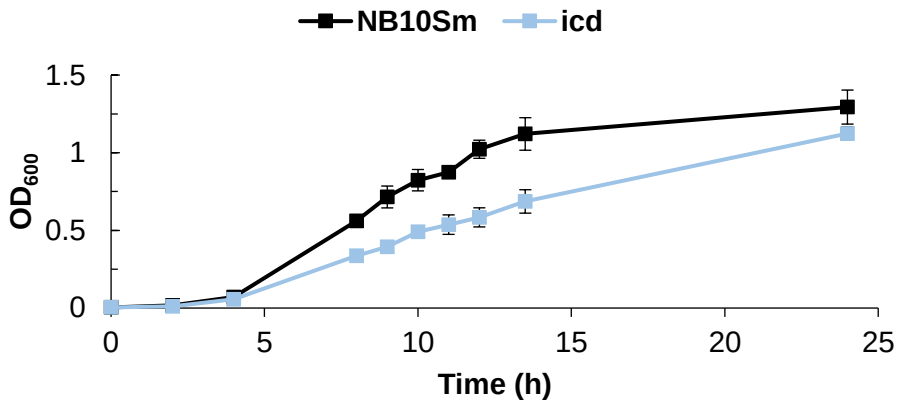
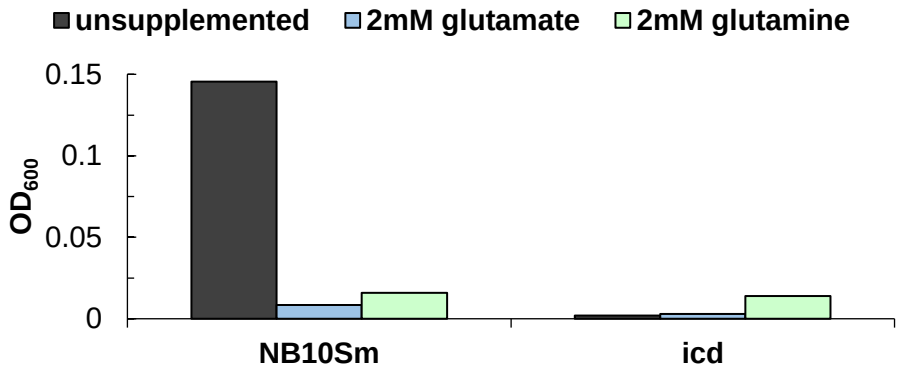

Glucose
Fructose
Fructose-1-P
fruA/B
fruP
Fructose-1,6-P
cra
Phosphoenolpyruvate
Acetyl-CoA
gltA
Oxaloacetate
Citrate
acn
mdh
Aspartate
Malate
Isocitrate
icd
fumA
Fumarate
α-Ketoglutarate
frdABCD
sucAB
sdhCDAB
Succinate
Succinyl-CoA
sucCD

Figure 2. Growth of *V. anguillarum* WT (NB10Sm) and the *icd* mutant (ES920) under various conditions. Growth curves of *V. anguillarum* strains NB10Sm and the *icd* mutant in LB20 at 27°C with shaking (200 rpm). At various time points after inoculation samples were taken for determination of optical density at 600 nm (OD_{600}). The data are the average of two independent experiments. Error bars represent 1 standard deviation. **B)** Final cell densities (OD_{600}) of *V. anguillarum* strains NB10Sm and the *icd* mutant after 24 h of growth in 3M plus 0.15% glucose supplemented with or without 2 mM glutamate or 2 mM glutamine. In each experiment cells grown overnight in LB20 were washed in NSS and used to inoculate the appropriate media. Cultures were incubated at 27^{o}C in a shaking water bath (200 rpm) and at various time points after inoculation samples were taken for determination of optical density at 600 nm (OD_{600}).

A


NB10Sm
icd
1.5
1
0.5
0
OD600
0
5
10
15
20
25
Time (h)

B


unsupplemented
2mM glutamate
2mM glutamine
0.15
0.1
0.05
0
OD600
NB10Sm
icd

C

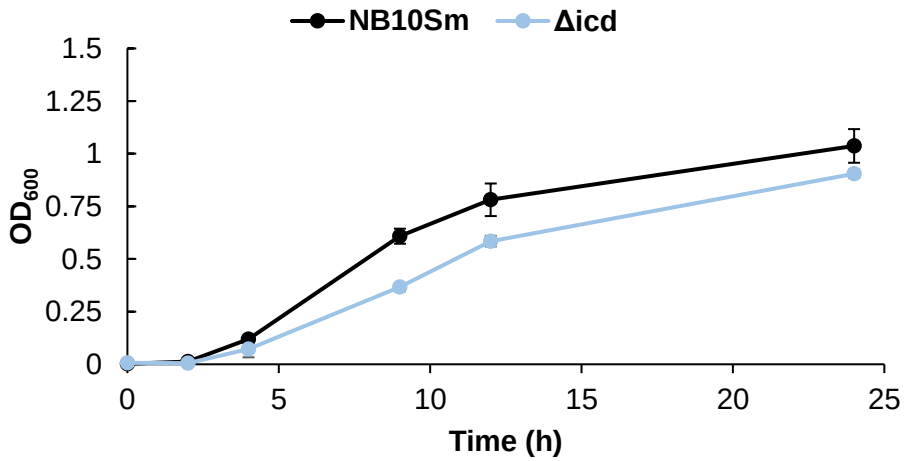
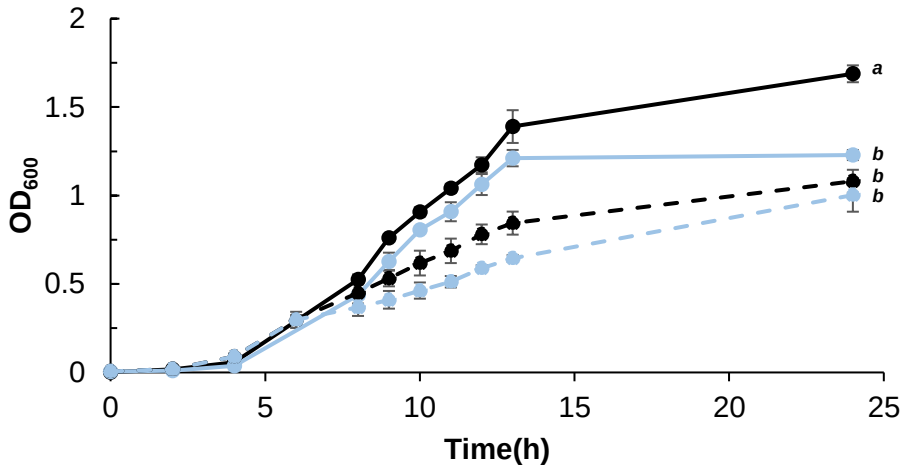

NB10Sm
Δicd
1.5
1.25
1
0.75
0.5
0.25
0
OD600
0
5
10
15
20
25
Time (h)

Figure 3. Growth of *V. anguillarum* WT (NB10Sm) and the *icd* mutant under various conditions. **A)** Growth curves of *V. anguillarum* NB10Sm (black) and the *icd* mutant (blue) in LB20 (dashed lines) or LB20 supplemented with 101.7 mM gluconate (solid lines). **B)** Growth curves of *V. anguillarum* NB10Sm (black) and the *icd* mutant (blue) in LB20 (dashed lines) or LB20 supplemented with 118 mM glutamate (solid lines). In each experiment cells grown overnight in LB20 were washed in NSS and used to inoculate the appropriate media. Cultures were incubated at 27^{o}C in a shaking water bath (200 rpm) and at various time points after inoculation samples were taken for determination of optical density at 600 nm (OD_{600}). The data are the average of two independent experiments. Different letters indicate statistical significance among groups ($P < 0.05$). Statistical analysis was based on data at 24 h. Error bars represent 1 standard deviation.

A


2
1.5
1
0.5
0
OD600
0
5
10
15
20
25
Time(h)
a
b
b
b

B

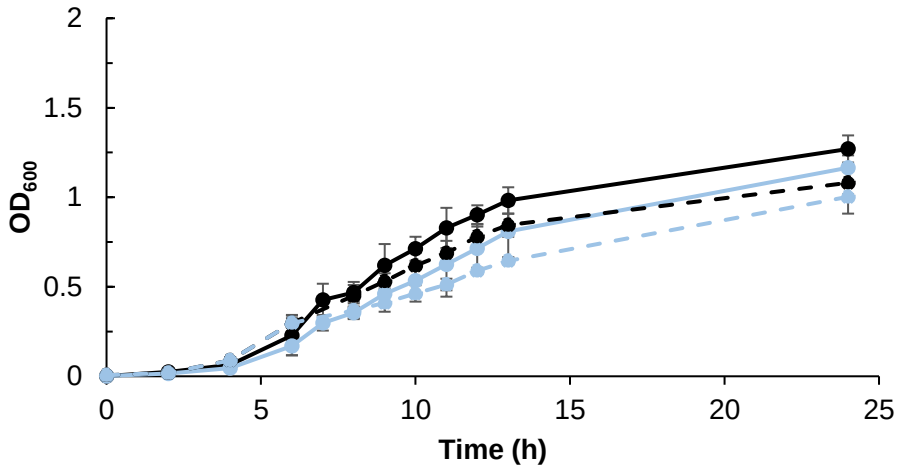

2
1.5
1
0.5
0
OD600
0
5
10
15
20
25
Time (h)

Figure 4. Growth curves of various *V. anguillarum* strains grown in LB20 at 27°C with shaking (200 rpm). At various time points after inoculation samples were taken for determination of optical density at 600 nm (OD_{600}). The data are the average of two independent experiments. Error bars represent 1 standard deviation.

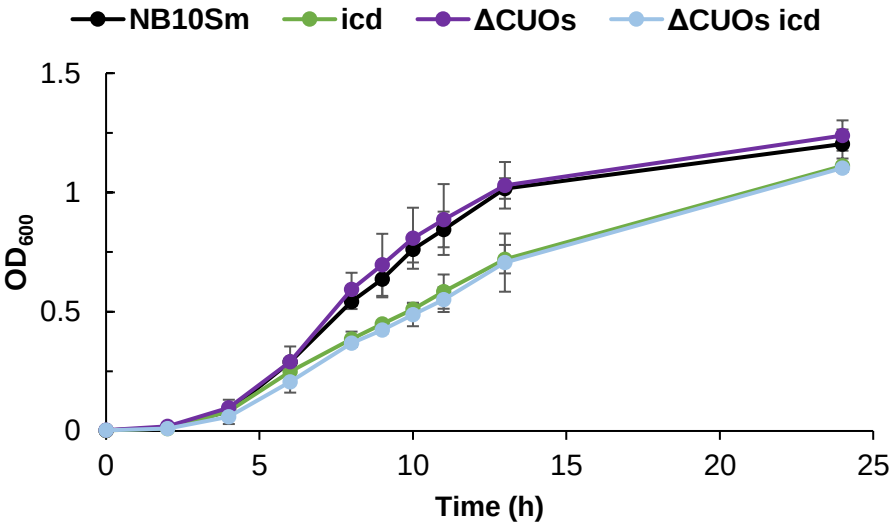
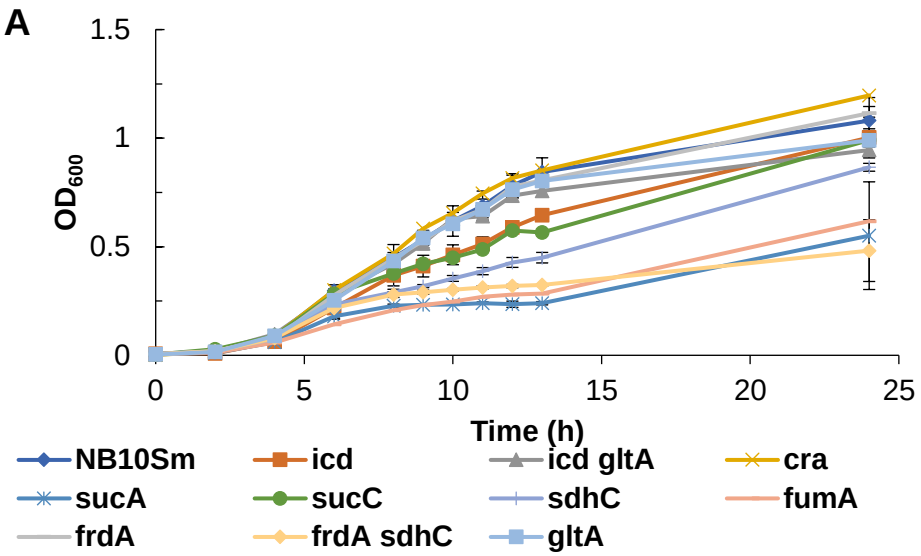

NB10Sm
icd
ΔCUOs
ΔCUOs icd
1.5
1
0.5
0
OD600
0
5
10
15
20
25
Time (h)

Figure 5. Growth of various *V. anguillarum* strains grown in LB20 at 27°C with shaking (200 rpm). **A)** Growth curve of various *V. anguillarum* strains. At various time points after inoculation samples were taken for determination of optical density at 600 nm (OD_{600}). **B**) Examination of the 24 h time point from Figure 5A. The data are the average of two independent experiments. Between marked strains and NB10Sm: * $P < 0.05$ and *** $P < 0.001$. Error bars represent 1 standard deviation.


A
1.5
1
0.5
0
OD600
0
5
10
15
20
25
Time (h)
NB10Sm
icd
icd gltA
cra
sucA
sucC
sdhC
fumA
frdA
frdA sdhC
gltA

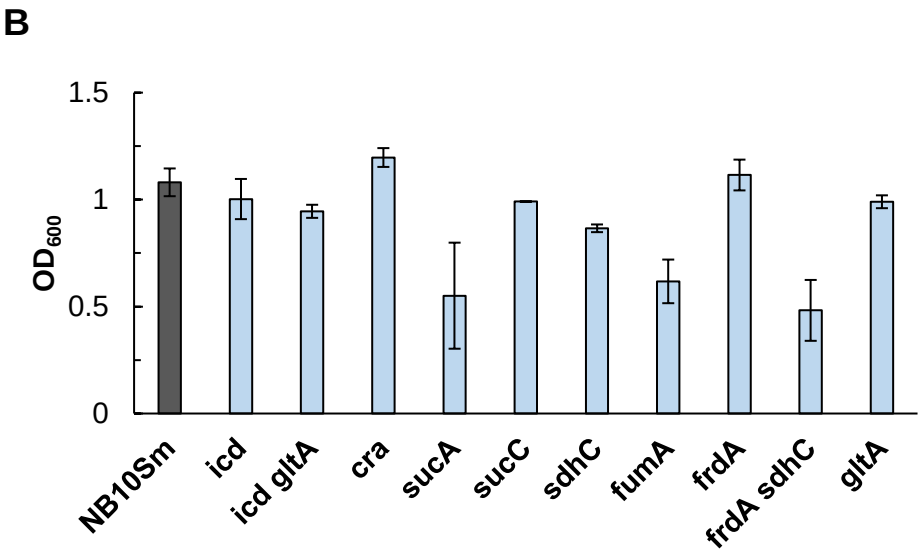

B
1.5
1
0.5
0
OD600
NB10Sm
icd
icd gltA
cra
sucA
sucC
sdhC
fumA
frdA
frdA sdhC
gltA

Figure 6. Growth of *V. anguillarum* WT (NB10Sm) the Δ*sucA* mutant and the Δ*frdA ΔsdhC* mutant under various conditions. **A)** Final cell densities (OD_{600}) of *V. anguillarum* strains NB10Sm, the Δ*sucA* mutant and the Δ*frdA ΔsdhC* mutant after 24 h or 48 h of growth in LB20. **B)** Final cell densities (OD_{600}) of *V. anguillarum* strains NB10Sm and the Δ*frdA ΔsdhC* mutant after growth for 24 h in LB20 with or without the addition of either succinic acid (118 mM), gluconate acid (118mM), malate acid (118 mM), or aspartate acid (118 mM). **C)** Final cell densities (OD_{600}) of *V. anguillarum* strains NB10Sm and the Δ*sucA* mutant after 24 h of growth in LB20 with or without the addition of either lysine (118 mM) or DL-α, ε-Diaminopimelic acid. In each experiment cells grown overnight in LB20 were washed in NSS and used to inoculate the appropriate media. Cultures were incubated at 27^{o}C in a shaking water bath (200 rpm) and at various time points after inoculation samples were taken for determination of optical density at 600 nm (OD_{600}). The data are the average of two independent experiments. Different letters indicate statistical significance among groups ($P < 0.05$). Between marked strains and NB10Sm grown in LB20: ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. Error bars represent 1 standard deviation. Error bars represent 1 standard deviation.

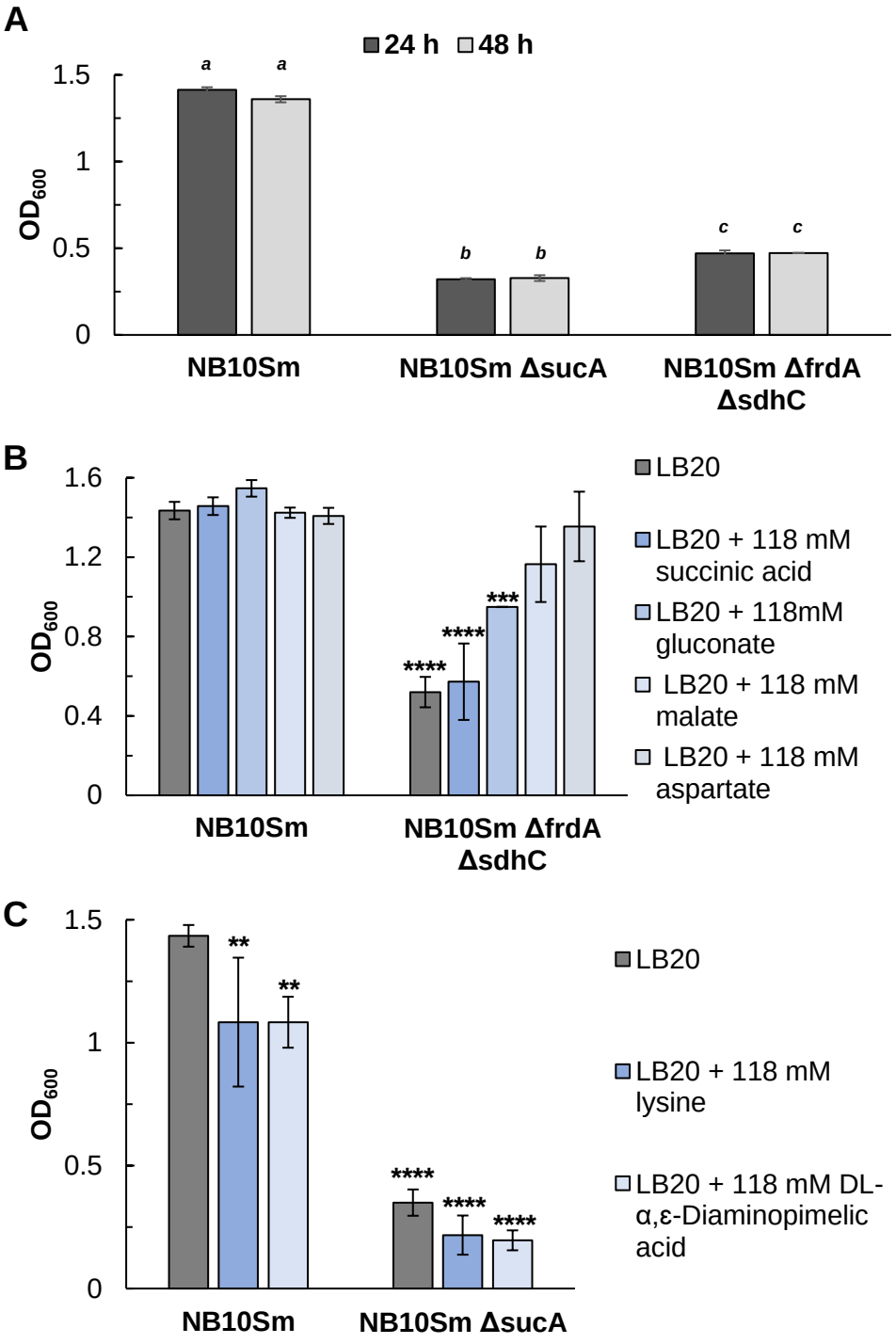
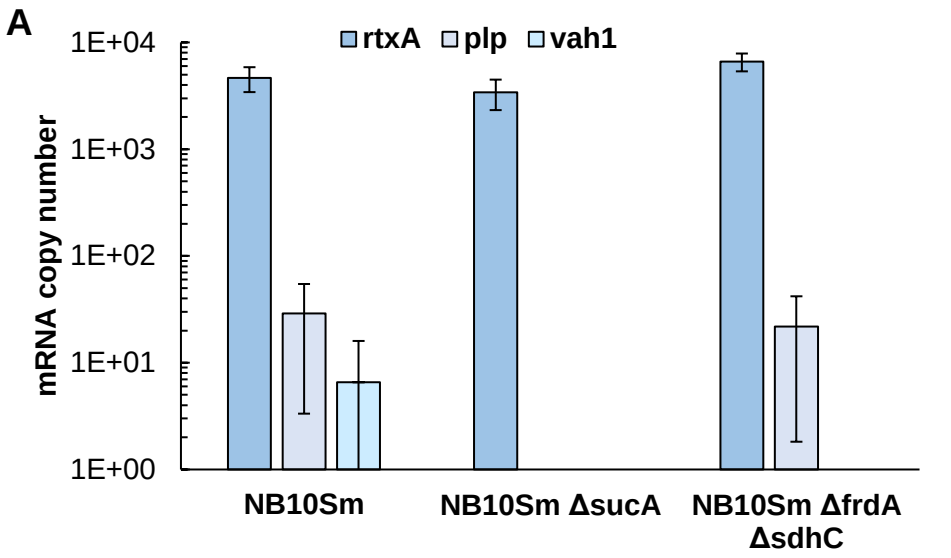

A
24 h
48 h
1.5
1
0.5
0
OD600
a
a
b
b
c
c
NB10Sm
NB10Sm ΔsucA
NB10Sm ΔfrdA ΔsdhC
B
1.6
1.2
0.8
0.4
0
OD600
****
****
***
NB10Sm
NB10Sm ΔfrdA ΔsdhC
LB20
LB20 + 118 mM succinic acid
LB20 + 118mM gluconate
LB20 + 118 mM malate
LB20 + 118 mM aspartate
C
1.5
1
0.5
0
OD600
**
**
****
****
****
NB10Sm
NB10Sm ΔsucA
LB20
LB20 + 118 mM lysine
LB20 + 118 mM DL-α,ε-Diaminopimelic acid

Figure 7. mRNA expression levels of various genes. **A)** Expression of *vah1*, *plp*, *rtxA* determined by qRT-PCR analysis of *V. anguillarum* wild-type (NB10Sm), the Δ*sucA* mutant and the Δ*frdA ΔsdhC* mutant during logarithmic (Log)-phase growth. **B)** Expression of *empA* determined by qRT-PCR analysis of *V. anguillarum* wild-type (NB10Sm), the Δ*sucA* mutant and the Δ*frdA ΔsdhC* mutant during stationary phase (Stat)-phase growth. The data presented are representative of two independent experiments. Each value is the average for six replicates. Between marked strains and NB10Sm: ** $P < 0.01$. Error bars represent 1 standard deviation.


A
rtxA
plp
vah1
mRNA copy number
1E+04
1E+03
1E+02
1E+01
1E+00
NB10Sm
NB10Sm ΔsucA
NB10Sm ΔfrdA ΔsdhC

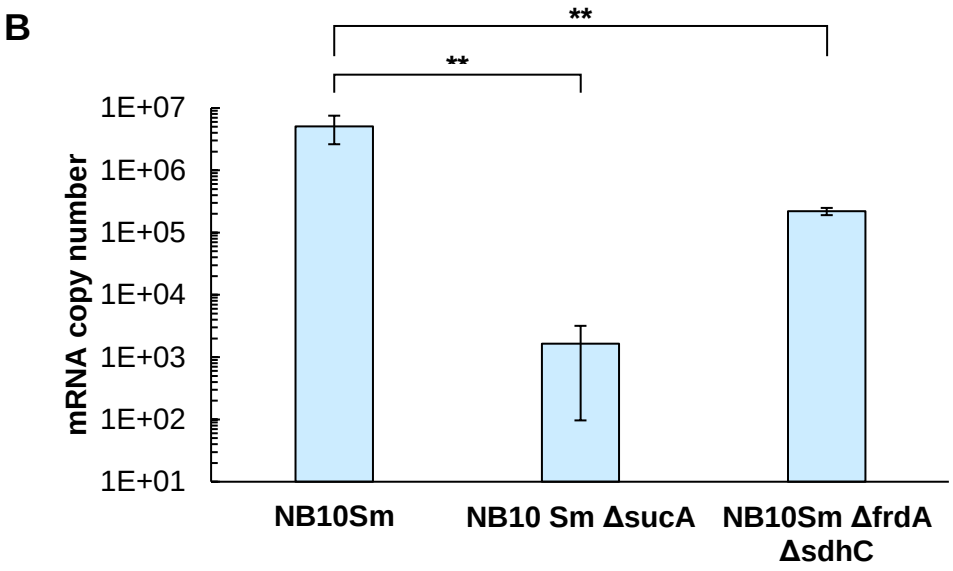

B
**
**
mRNA copy number
1E+07
1E+06
1E+05
1E+04
1E+03
1E+02
1E+01
NB10Sm
NB10 Sm ΔsucA
NB10Sm ΔfrdA ΔsdhC

Figure S1. Cell density determined by viable count vs. OD_{600} of *V. anguillarum* NB10Sm. The line indicates linear regression.

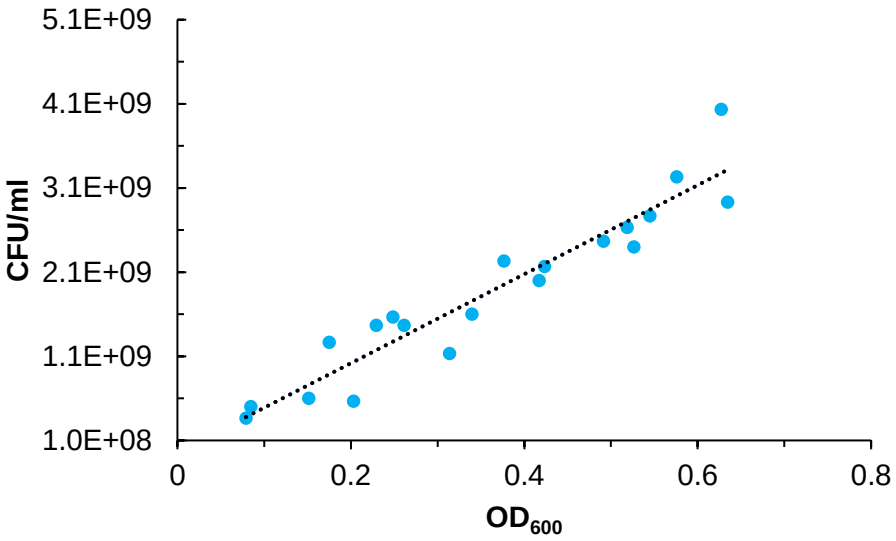

5.1E+09
4.1E+09
3.1E+09
2.1E+09
1.1E+09
1.0E+08
CFU/ml
0
0.2
0.4
0.6
0.8
OD600

Figure S2. Graphical representation of the CUO region (blue arrows) and flanking genes (green arrows) in the genomes of *V. anguillarum* **A)** NB10 and **B)** M93Sm. Yellow arrows represent open reading frames encoding hypothetical proteins.

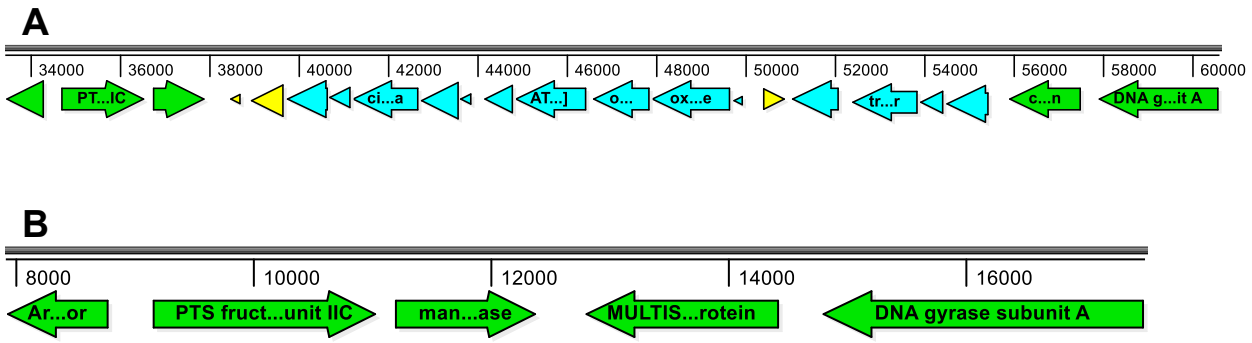

A
34000
36000
38000
40000
42000
44000
46000
48000
50000
52000
54000
56000
58000
60000
PT...IC
ci...a
AT...]
o...
ox...e
tr...r
c...n
DNA g...it A
B
8000
10000
12000
14000
16000
Ar...or
PTS fruct...unit IIC
man...ase
MULTIS...rotein
DNA gyrase subunit A

Figure S3. Graphical representation of a suicide vector inserting into the *sucA* gene. **A)** First a crossover event occurs in the genome and the suicide vector inserts in the 5' region of *sucA.* **B)** If the cells are grown without selection a second cross over event can occur that will **C)** resolve the merodiploid and restore the wild type *sucA.* Figure not drawn to scale.

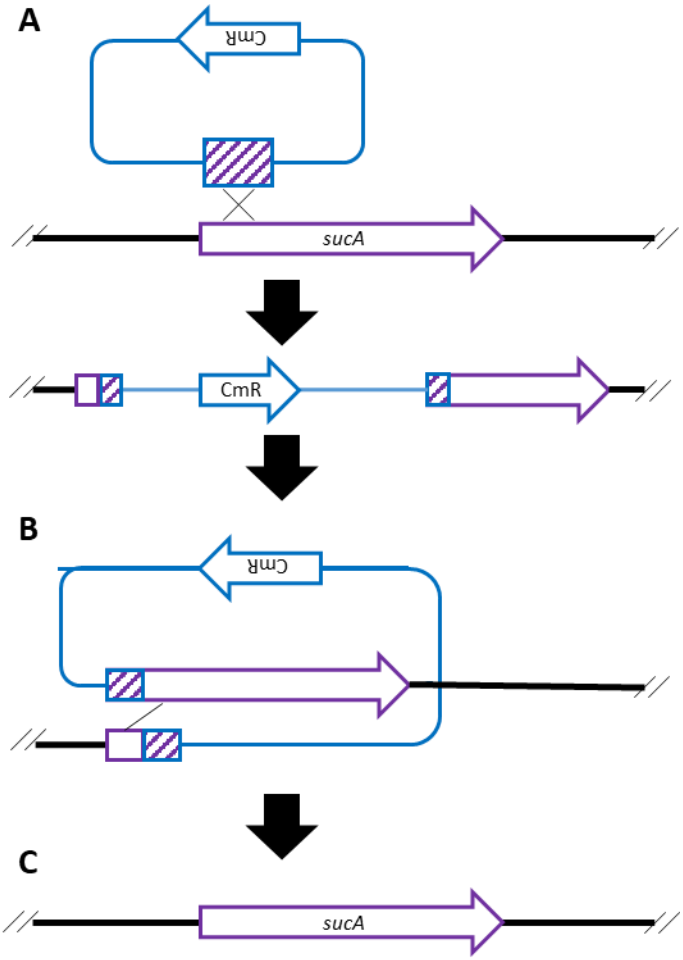
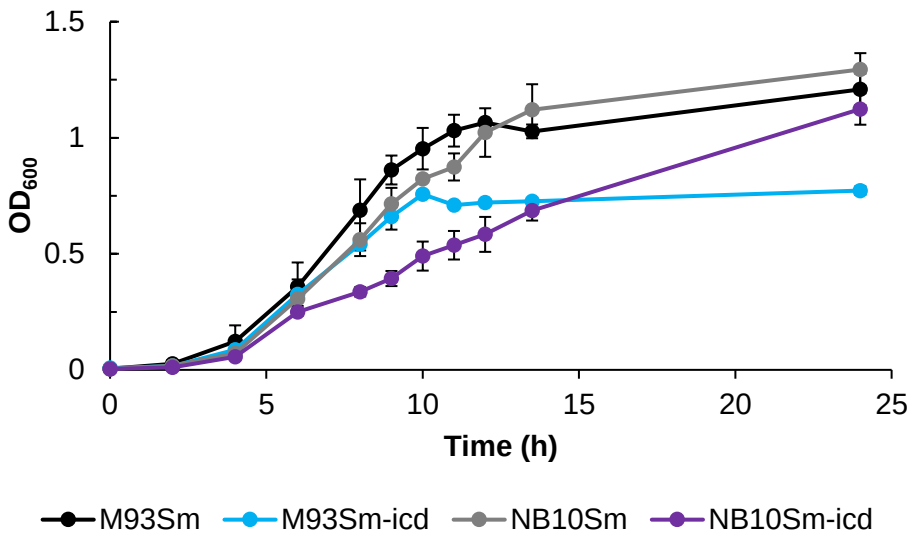

A
CmR
sucA
CmR
B
CmR
C
sucA

Figure S4. Growth curves of *V. anguillarum* M93Sm, NB10Sm, and their derived *icd* mutant strains grown in LB20 at 27°C with shaking (200 rpm). At various time points after inoculation samples were taken for determination of optical density at 600 nm (OD_{600}). The data are the average of two independent experiments. Error bars represent 1 standard deviation.


1.5
1
0.5
0
OD600
0
5
10
15
20
25
Time (h)
M93Sm
M93Sm-icd
NB10Sm
NB10Sm-icd

References

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Appendix-I

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Draft genome of the marine pathogen *Vibrio coralliilyticus* RE22

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Running Head: Genome of marine pathogen *Vibrio coralliilyticus* RE22

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Vibrio coralliilyticus RE22 is a causative agent of vibriosis in larval bivalves. We report the draft genome of *V. coralliilyticus* RE22 and describe additional virulence factors, which may provide insight into the mechanism of pathogenicity.

Vibrio coralliilyticus RE22 (formally *Vibrio tubiashii* RE22) is a marine pathogen and a causative agent of vibriosis in larval bivalves (1). Disease is characterized by high mortality rates leading to severe loss of production in shellfish hatcheries (2-4). Currently, only two proteases (VtpA and VtpB) and one hemolysin (VthA) have been characterized in RE22 (5-7). To better understand mechanisms of pathogenicity, it is necessary to discover additional potential virulence factors. Here we announce the draft genome sequence of *V. coralliilyticus* RE22 and selectively describe some potential virulence factors.

V. coralliilyticus RE22Sm (a spontaneous mutant resistant to streptomycin) was grown overnight in yeast-peptone broth supplemented with 3% NaCl (YP30) at 27°C in a shaking water bath. Genomic DNA was isolated using the Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's instructions except DNA was resuspended into 100 µl of a 2mM Tris-HCl, pH 8solution. DNA was sequenced at the Rhode Island Genomics Sequencing Center using an Illumina MiSeq Sequencer. Reads were trimmed using the CLC Genomics Workbench (v8.0.1) for quality, ambiguous base pairs, adapters, duplicates and size resulting in 7,602,646 paired-end and mate-paired reads averaging 235.84 bp in size. Reads were assembled using the de novo assembly algorithm of CLC Genomics Workbench and SPAdes Genomic Assembler (v3.1.1) (8). Contigs with an average coverage above 110 reads were joined using the CLC Microbial Genome Finishing module using *V. coralliilyticus* OCN014 as a reference genome. In

total, the draft genome is composed of five contigs. Three contigs totaling 3.46 Mbp and having an average G+C content of 46% mapped to chromosome 1 of *V. coralliilyticus* OCN014. The complete chromosome 2 is represented by one 1.90 Mbp contig with a G+C content of 45%. A megaplasmid is represented by one 0.32 Mbp contig with a G+C content of 50%. The draft genome was annotated using Rapid Annotation using Subsystem Technology (RAST) and resulted in 5234 open reading frames (9-11).

The genome of *V. coralliilyticus* RE22 encodes two extracellular metalloproteases besides the previously described *vtpA* and *vtpB*. One protease shows similarity to *epp* in *Vibrio anguillarum* (12) while the other contains a domain conserved in M4 family of metalloproteases (13-17). In addition to *vthA*, three putative hemolysin/cytolysin genes were discovered. A putative MARTX toxin operon encoding three T1SS transport proteins, a MARTX toxin, and a hypothetical protein is on the megaplasmid. Unlike typical MARTX toxin gene clusters, the transporter genes are not transcribed divergently from the MARTX toxin (18). Instead they seem to be in the MARTX operon, upstream of the MARTX toxin gene. Unlike most MARTX toxin gene clusters, no *rtxC* (acyltransferase) is present in the operon. Additional putative hemolysins include a phospholipase/hemolysin located on chromosome 2 that shows similarity to *plp* in *V. anguillarum* (19) and a hemolysin annotated as *hlyA* located on chromosome 1 that shows similarity to *vah1* in *V. anguillarum* (20).

This Whole Genome Shotgun project has been deposited in DDBJ/ENA/GenBank under the accession no. LGLS00000000. The version described in this paper is the first version, LGLS01000000.

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Appendix-II

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Draft genome sequence of the emerging bivalve pathogen *Vibrio tubiashii* subsp. *europaeus*.

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Running title: Draft genome of *Vibrio tubiashii* subsp. *europaeus*.

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Vibrio tubiashii subsp. *europaeus* is a bivalve pathogen isolated during episodes of mortality affecting larval cultures in different shellfish hatcheries. Here we announce the draft genome of the type strain PP-638 and describe potential virulence factors, which may provide insight into the mechanism of pathogenicity.

Vibrio tubiashii subsp. *europaeus* is an emerging bivalve pathogen identified recently as the etiological agent responsible of larval and spat mortalities in clam, oyster and abalone cultures detected in Spanish and French hatcheries (1, 2). This pathogen is a causative agent of vibriosis inducing mass mortalities and important economic losses, representing the main bottleneck for the production process in shellfish aquaculture (1, 2).

V. tubiashii subsp. *europaeus* PP-638^T (= CECT 8136^T= DSM 27349^T) was originally isolated from seawater in a culture tank of flat oyster (*Ostrea edulis*) during an episode of larval mortality detected in a shellfish hatchery (Galicia, NW Spain) (1). DNA was isolated from *V. tubiashii* subsp. *europaeus* PP-638^T grown up overnight in YP30 using the Wizard Genomic DNA Purification kit (Promega) following the manufacturer's instructions except DNA was resuspended in 2mM Tris-HCl Buffer (Bio Basic). Genomic DNA was sequenced using an Illumina MiSeq at the Rhode Island Genomics and Sequencing Center at the University of Rhode Island. Reads were trimmed using the CLC Genomics Workbench (v8.5.1) for quality, ambiguous nucleotides and adapters. 2,943,708 paired-end and 3,234,516 mate-paired reads providing 199× coverage were assembled using Spades (v3.1.1) using the default parameters (1). Contigs were filter based on 34× coverage and 4000 bp length resulting in ten contigs with an N50 of

1,788,614 and an average G + C content of 45.37%. The assembly was mapped to *Vibrio tubiashii* ATCC 19109 using the CLC Microbial Genome Finishing module and resulted in six contigs mapping to chromosome 1, one complete contig representing chromosome 2, one complete contig representing the p251-like megaplasmid and one contig mapping to the p57-like plasmid (2). One 4,885 bp contig did not map to the reference genome. The draft genome was submitted to Rapid Annotations using Subsystems Technology (RAST) for annotation resulting in 5157 open reading frames (3-5).

Encoded on chromosome 2 of the *V. tubiashii* subsp. *europaeus* PP-638^T genome is a putative metalloprotease that has a 75% similarity to VtpA found in *Vibrio coralliilyticus* RE22 (6). Another protease that has a 71% similarity to Epp in *Vibrio anguillarum* M93Sm is encoded on chromosome 2 (7). There are three putative hemolysins and phospholipases encoded in the genome. One hemolysin located on chromosome 2 has a 67% similarity to Plp in *V. anguillarum* M93Sm (8, 9). In *V. anguillarum* M93Sm, *plp* is divergently transcribed from the pore-forming hemolysin/cytolysin *vah1* (9). In *V. tubiashii* subsp. *europaeus* PP-638^T, the Plp homolog is also divergently transcribed away from a pore-forming cytolysin, though it has a 42% similarity to aerolysin in *Aeromonas eucrenophila*, not *vah1* (NCBI Reference Sequence: WP_042642875.1). The genome encodes two secretion systems (T3SS and T6SS) that are used to deliver effecter molecules directly into the host. The T3SS secreted virulence factor has a domain that is similar to the GTPase-activating domain found on YopE from *Yersinia pestis* (10-14). While the T6SS structural components are encoded on the p251-like megaplasmid, the protein responsible for forming the puncturing tip of the T6SS

secretion system, VgrG, appears to be encoded by two genes. One VgrG-encoding gene is on Chromosome 1 and the second is on Chromosome 2.

Nucleotide sequence accession numbers. This Whole Genome Shotgun project has been deposited in DDBJ/EMBL/GenBank under the accession number LUAX00000000. The version described in this paper is the first version LUAX01000000.

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